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NAME OF SUPERVISOR/NOM DU DIRECTEUR DE THÈSE Dr. David Nash

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NUTRITIONAL STUDIES ON 5-FLUORO-2'-DEOXYURIDINE
AND NUCLEIC ACID BASES AND NUCLEOSIDES IN
DROSOPHILA MELANOGASTER

by



MAHMOUD HAMDİ el KOUNI

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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EDMONTON, ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Nutritional Studies on 5-Fluoro-2'-Deoxyuridine and Nucleic Acid Bases and Nucleosides in *Drosophila melanogaster*, submitted by Mahmoud Hamdi el Kouni, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

..... David J. Noel
Supervisor

..... R. M. Gossel
.....

..... R. B. Hodgkins
.....

..... Kenneth L. Roy
.....

..... A. R. Palmer
.....

..... C. H. Kemp
External Examiner

DATED Feb. 18 1977

ABSTRACT

Growth responses of *Drosophila melanogaster* larvae on defined medium containing purine and pyrimidine bases, nucleosides and deoxynucleosides have been measured at doses from $3.16 \times 10^{-5} \text{M}$ to $3.16 \times 10^{-2} \text{M}$. Purine compounds are shown to be more toxic than pyrimidine compounds. A number of mechanisms are proposed for the toxicity of these compounds.

Studies on FUdR show that the analogue at the concentration of 10^{-6}M kills the larvae. The addition of uridine ($5 \times 10^{-3} \text{M}$) reduces the sensitivity to the analogue 100-fold. Neither uridine ($5 \times 10^{-3} \text{M}$) nor RNA (0.4%) has a similar effect. It has been concluded that the effects of FUdR at low doses (10^{-6} to 10^{-5}M) are due to inhibition of the enzyme thymidylate synthetase while at higher doses (10^{-4}M and above) it includes effects on RNA and, possibly, *de novo* pyrimidine biosynthesis.

Medium shift experiments, between media with and without FUdR, indicate a prolonged period during larval life when the analogue is effective, both under conditions when DNA synthesis is inhibited and when other aspects of metabolism are affected.

The responses of larvae to FUdR and to other pyrimidines and purines, with the exception of deoxycytidine, in the presence of FUdR reflect similarities of nucleotide metabolism in *Drosophila* and in other organisms. They also indicate the potential sensitivity of nutritional manipulation for such studies.

Difficulties were encountered in obtaining FUDR resistant mutants. After EMS mutagenesis (4.3 mM), no mutants were selected at doses higher than 1.5×10^{-6} M FUDR. At 1.5×10^{-6} M FUDR, in the presence of thymine (5×10^{-3} M), 14 mutant strains were isolated. However, all strains exhibited a low level of resistance and were phenotypically unstable with the exception of one strain, A37. The productivity of A37 is reduced in the presence of dTMP sources, even in the absence of FUDR. It is suggested that this mutant has an elevated level of thymidylate synthetase activity.

The rest of the mutants could be leaky mutants in the thymidylate synthetase or thymidine kinase genes, or could have slightly elevated thymidylate synthetase activity or could be transport mutants.

Preliminary genetic studies indicate that all of the mutants are recessive, complement one another and are of autosomal origin. Four mutants were mapped to one or the other large autosome.

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ABBREVIATIONS

A	adenine
ADP	adenosine diphosphate
AdR	deoxyadenosine
AMP	adenosine monophosphate
AR	adenosine
APRT	adenine phosphoribosyltransferase
ATP	adenosine triphosphate
BuDR	5-bromo-2'-deoxyuridine
C	cytosine
CDP	cytidine diphosphate
CdR	deoxycytidine
CMP	cytidine monophosphate
CR	cytidine
CTP	cytidine triphosphate
dADP	deoxyadenosine diphosphate
dAMP	deoxyadenosine monophosphate
dATP	deoxyadenosine triphosphate
dCDP	deoxycytidine diphosphate
dCMP	deoxycytidine monophosphate
dCTP	deoxycytidine triphosphate
DHFA	dihydrofolic acid
dGDP	deoxyguanosine diphosphate
dGMP	deoxyguanosine monophosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid

dR-1-P	deoxyribose-1-phosphate
dR-5-P	deoxyribose-5-phosphate
dTDP	thymidine diphosphate
dTMP	thymidine monophosphate
dTTP	thymidine triphosphate
dUDP	deoxyuridine diphosphate
dUMP	deoxyuridine monophosphate
dUTP	deoxyuridine triphosphate
EMS	ethyl methanesulfonate
FC	5-fluorocytosine
FCdR	5-fluoro-2'-deoxycytidine
FCR	5-fluorocytidine
FdUDP	5-fluoro-2'-deoxyuridine diphosphate
FdUMP	5-fluoro-2'- deoxyuridine monophosphate
FU	5-fluorouracil
FUDP	5-fluorouridine diphosphate
FudR	5-fluoro-2'-deoxyuridine
FUMP	5-fluorouridine monophosphate
FUR	5-fluorouridine
FUTP	5-fluorouridine triphosphate
G	guanine
GDP	guanosine diphosphate
GdR	deoxyguanosine
GMP	guanosine monophosphate
GPRT	guanine phosphoribosyltransferase
GR	guanosine
GTP	guanosine triphosphate

II	hypoxanthine
HdR	deoxyinosine
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
HPRT	hypoxanthine phosphoribosyltransferase
IIR	inosine
IMP	inosine monophosphate
ITP	inosine triphosphate
Mg	magnesium
NAD ⁺	nicotinamide adenine dinucleotide
P _i	inorganic phosphate
PP _i	pyrophosphate
PPRT	pyrimidine phosphoribosyltransferase
PRPP	phosphoribosyl pyrophosphate
Pur	purine base
R-1-P	ribose-1-phosphate
R-5-P	ribose-5-phosphate
RNA	ribonucleic acid
SAMP	adenylo-succinate
T	thymine
TdR	thymidine
THFA	tetrahydrofolic acid
U	uracil
UDP	uridine diphosphate
UdR	deoxyuridine
UMP	uridine monophosphate
UPRT	uracil phosphoribosyltransferase
UTP	uridine triphosphate

X	xanthine
XMP	xanthosine monophosphate
XR	xanthosine

INTRODUCTION

1. THE PROBLEM

One of the major difficulties in studying developmental genetics in higher organisms is the isolation of mutants that affect specific enzymes or processes. Mutation frequencies at any given locus are quite low even after enhancement with potent mutagens; hence, thousands of individuals must be screened before such mutants can be detected. Furthermore, mutants in fundamental developmental processes would probably be lethal and thus difficult to study. These factors are further complicated by the diploidy of most higher organisms, necessitating the laborious testing of individual progeny lines of every mutagenized organism to obtain such rare mutations. In this study an attempt is made to avoid some of these difficulties by the use of metabolite analogue toxicity as a screening agent, a method which might minimize these problems, since only mutants would survive the screen.

Nucleotide metabolism was chosen since it provides an excellent opportunity for such studies. The normal function of genes governing these pathways and their coordination with one another was elucidated with the aid of auxotrophs and analogue resistant mutants in microorganisms. Such mutants proved to be powerful tools in resolving ambiguities about the utilization of certain compounds, the existence of particular enzymes, and the correct sequence of steps in specific pathways. These mutants were also useful in comparing the *in vivo* and *in vitro* functions of certain enzymes. For examples and details of such studies see O'Donovan and Neuhaud (1970) and Beck *et al.* (1972a).

These achievements have not, as yet, been matched in multicellular organisms. Difficulties arose mainly from the lack of appropriate conditions under which a selection scheme for auxotrophy and analogue resistance could be set up.

The success of Sang (1956) in creating a defined medium for the fruit fly, *Drosophila melanogaster* and the ability of this organism to develop in the absence of nucleic acid precursors indicate its capacity for *de novo* nucleotide biosynthesis. This fact, in addition to the existence of a powerful mutagen, ethyl methanesulfonate (EMS), and the wealth of knowledge regarding fly genetics, suggests the possibility for the isolation of nucleoside auxotrophs and nucleoside or base analogue resistant mutants in *Drosophila*. Successful attempts to isolate auxotrophs by Vyse and Nash (1969), Falk and Nash (1974 a and b), Naguib (1976) and Naguib and Nash (1976) have so far led to the isolation of 40 nucleoside auxotrophs in at least eight different loci in this organism. Norby (1970) discovered, also, that mutants at the rudimentary locus are pyrimidine requirers.

Successful isolation of nucleoside auxotrophs in *Drosophila* generates interest in exploring the possibility of using resistance to purine and pyrimidine analogues to screen for more mutants that affect nucleotide metabolism in this organism. This method is useful in isolating mutants in specific enzymes in microorganisms and mammalian tissue culture, since resistance to such analogues generally has quite specific causes. Analogue resistance techniques have been used in *Drosophila* in attempts to isolate mutants in alcohol dehydrogenase (Sofer and Hatkoff, 1972; O'Donnell *et al.*, 1975) and dopadecarboxylase

(Sparrow and Wright, 1974; Sherald and Wright, 1974) and mutants resistant to juvenile hormone analogues (Arking and Vlach, 1976). It has also been used by Duke and Glassman (1968) to investigate the mechanism of FU toxicity in the fly. Similar resistant mutants would undoubtedly help in understanding the mechanism by which *Drosophila* can utilize exogenously supplied purines and pyrimidines. Such knowledge, besides being crucial to understanding nucleotide metabolism in general, should also facilitate the isolation of more mutants in the various steps of these metabolic pathways and elucidate the mechanism of action of the different purine and pyrimidine analogues. Furthermore the study of drug resistance in a well studied whole organism like *Drosophila* should complement studies using cultured cells. It has been reported that hypoxanthine-guanine phosphoribosyltransferase (HGPRT) deficiency selected as drug resistant has no deleterious effect on cultured cells but is known to cause severe abnormalities in persons suffering from such deficiency (Seegmiller, 1972).

In studies on analogues, as well as supplementation studies on *Drosophila* nucleoside auxotrophs, it is commonly useful to know the levels of nucleotide precursors tolerable by the wild type. Although studies on the utilization of nucleic acid precursors in *Drosophila* started as early as the 1940's (Wilson, 1942, 1943a, b, c and 1944), controversies about the utilization and toxicity for such compounds are numerous in the literature. For example, Sang (1957) showed that dietary cytidine does not stimulate growth and hence concluded that *Drosophila* cannot utilize cytidine; on the other hand, Hinton (1956) demonstrated that cytidine improved survival from 74% to 89% and

stated that cytidine is utilized. Similarly, Burnet and Sang (1963) using growth rate and survival as criteria for utilization concluded that thymine is not utilized, although Goldsmith and Harnly (1950) had shown that this base reduced the toxicity caused by dietary aminopterin and suggested that thymine is used as a thymidylate source when thymidylate synthetase [E.C.2.1.1.b] is inhibited by the antimetabolite. Despite such controversies no report of a systematic study of the response of *Drosophila* to dietary purines and pyrimidines has yet been published and, as a preliminary matter, this aspect of nutrition is also treated in this thesis.

II. UTILIZATION OF BASES AND NUCLEOSIDES FOR NUCLEOTIDE METABOLISM

The biochemistry, regulation and genetics of the *de novo* pathways of purine and pyrimidine nucleotides in different organisms are now well documented (for review see O'Donovan and Neuhaard, 1970; Henderson and Paterson, 1973; Naguib, 1976).

Although *de novo* pathways are major routes by which purine and pyrimidine nucleotides are synthesized, alternative pathways for the synthesis of these compounds are known. These "salvage" mechanisms are used either to reutilize preexisting nucleotides and their derivatives or to utilize exogenous sources of these compounds.

The relative importance of the salvage pathway was not fully recognized until the isolation of the first auxotrophs (see Magasanik, 1962) and the finding that some tissues such as bone marrow, leukocytes, erythrocytes, blood platelets and intestinal mucosa (for

references see Murray, 1971; Mackinnon and Deller, 1973) and organisms like *Tetrahymena geleii* (Kidder and Dewey, 1948), the majority of *Lactobacilli* (O'Donovan and Neuhard, 1970) and brine shrimp (van Denbos and Finamore, 1974) are deficient in the *de novo* synthetic capacity. In these cases it was clearly shown that a cell or an organism can sustain life possessing only the "salvage" pathways. The significant role of salvage pathways was further realized by the finding that the absence or change of some of its steps are associated with severe abnormalities in man, such as Lesch-Nyhan Syndrome (Seegiller *et al.*, 1967), gout (Kelley *et al.*, 1967) and immunodeficiency (for references see Ullman *et al.*, 1976).

Although by the late 1940's investigators had established the existence of *de novo* biosynthesis of both purine and pyrimidine nucleotides very little was known about the salvage pathways. Difficulties in interpretation of results available at that time were attributable primarily to the presence of the *de novo* pathways and to the inherent limitations of the classical nutritional studies which had been employed. In early experiments animals were reared on diets low in or free from purines and pyrimidines: a basal level of urinary nitrogen excretion was established under such conditions. If the amount of nitrogen excretion rose upon administration of purines or pyrimidines, the source of the additional nitrogen was identified to establish whether it was due to the presence of the compound administered *per se* or to any of its metabolites. The most successful experiments were in systems where a large amount of the compound could be administered without exerting a toxic effect on the organisms studied. However results



from such experiments - where a relatively slight increase in urinary nitrogen was most commonly observed - could rarely be interpreted with ease. Although the results of such experiments provided insight into catabolic products, they never really proved that preformed purines and pyrimidines acted as precursors for nucleic acid metabolism. For a review of such experiments, see Cerecedo (1927), Emerson and Cerecedo (1930) and Cerecedo and Allen (1934).

Nutritional studies were also carried out with *Drosophila*, where the utilization of purines and pyrimidines was mostly inferred either from developmental responses of larvae to dietary supplements (see Tables 1 and 2) or from the protection that these compounds will afford in the presence of antimetabolites (Goldsmith and Harnly, 1950; Schultz, 1956; Bos *et al.*, 1969; Rizki and Rizki, 1973).

The conclusive evidence for the utilization of preformed purines and pyrimidines as nucleic acid precursors waited until isotopically labelled compounds were available and for the later discovery of auxotrophs with absolute requirements for purine and pyrimidine compounds.

When isotopically labelled compounds were first used, it seemed that animals were unable to utilize either purine or pyrimidine bases. Plentl and Schoenheimer (1944) failed to show the incorporation of ¹⁵N-guanine, uracil or thymine into rat nucleic acids. Similarly, cytosine (Bendich *et al.*, 1949) hypoxanthine and xanthine (Getler *et al.*, 1949) appeared not to be incorporated. It was concluded that neither of these compounds was utilized for nucleic acid synthesis. Brown *et al.* (1948) confirmed the negative guanine results mentioned above, but

Table 1. Summary of Literature on the Effects on Survivorship and Development Rate Amongst Wild Type *Drosophila* Larvae Supplemented with Pyrimidines and Pyrimidine Nucleosides

Compound	Concentration ⁽¹⁾ (M)	Survivorship ⁽²⁾	Effect on Rate of Development	Medium ⁽³⁾	Strain	Sampling Technique ⁽⁴⁾	Reference
Uracil	2.0×10^{-2}	not toxic	slows	Y	Swedish-b	ET	Wilson (1943b)
	9.8×10^{-4}	0.68	-	S	Oregon-R	ET	Hinton et al. (1951)
	9.8×10^{-4}	1.26	accelerates	S	Oregon-R	ET	Hinton (1956)
	2.3×10^{-3}	-	slows	S	Oregon-S	LT	Sang (1957)
Uridine	6.75×10^{-4}	1.06	slows	S	Oregon-R	ET	Hinton (1956)
	2.3×10^{-3}	-	slows	S	Oregon-S	LT	Sang (1957)
Deoxyuridine-----NOT STUDIED-----							
Cytosine	1.0×10^{-2}	not toxic	no effect	Y	Swedish-b	ET	Wilson (1943c)
	9.9×10^{-4}	1.19	slows	S	Oregon-R	ET	Hinton (1956)
	2.3×10^{-3}	-	slows	S	Oregon-S	LT	Sang (1957)
Cytidine	6.8×10^{-4}	1.19	-	S	Oregon-R	ET	Hinton et al. (1951)
	6.8×10^{-4}	1.20	slows	S	Oregon-R	ET	Hinton (1956)
	2.3×10^{-3}	-	slows	S	Oregon-S	LT	Sang (1957)
	6.8×10^{-4}	17.67	accelerates	S	Oregon-X	LT	Ellis (1939)
Deoxycytidine-----NOT STUDIED-----							
Thymine	2.0×10^{-2}	lethal	slows	Y	Swedish-b	ET	Wilson (1944)
	9.5×10^{-4}	0.41	slows	S	Oregon-R	ET	Hinton (1956)
	2.3×10^{-3}	-	slows	S	Oregon-S	LT	Sang (1957)
Thymidine	1.0×10^{-2}	0.78	-	Y	Groningen 67	ET	Boe et al. (1969)
	6.6×10^{-4}	0.22	-	YD	Stammes & Wurde	AD	Wolf (1971)

¹Concentrations were converted to Molarity for easier comparison; the highest concentration used by an author is reported.

²Estimated as relative to controls.

³Y: Yeast, S: Basal defined medium (Schultz et al., 1946), S: Sang's medium (Sang, 1956) YD: Yeast deficient.

⁴ET: Egg transfer, LT: Larval transfer, AD: Adult Oviposition.

Table 2. Summary of Literature on the Effects of Survivorship and Development Rate Amongst Wild Type *Drosophila* Larvae Supplemented with Purines and Purine Nucleosides

Compound	Concentration ⁽¹⁾ (M)	Survivorship ⁽²⁾	Effect on Rate of Development	Medium ⁽³⁾	Strain	Sampling ⁽⁴⁾ Technique	Reference
Hypoxanthine	8.1×10^{-4}	0.88	accelerates	S	Oregon-R	ET	Hinton (1956)
	2.3×10^{-3}	-	accelerates	S	Oregon-S	LT	Sang (1957)
Inosine	8.15×10^{-4}	0.98	accelerates	S	Oregon-R	ET	Hinton (1956)
	2.3×10^{-3}	-	accelerates	S	Oregon-S	LT	Sang (1957)
	2.4×10^{-3}	0.25	slows	SM	Riverside	LT	Geer (1963)
Xanthine	7.2×10^{-4}	0.70	accelerates	S	Oregon-R	ET	Hinton (1956)
	2.3×10^{-3}	-	accelerates slightly	S	Oregon-S	LT	Sang (1957)
Xanthosine	5.8×10^{-4}	0.74	accelerates	S	Oregon-R	ET	Hinton (1956)
	2.3×10^{-3}	-	accelerates slightly	S	Oregon-S	LT	Sang (1957)
Adenine	2.0×10^{-3}	lethal	slows	Y	Swedish-b	ET	Wilson (1942)
	4.0×10^{-3}	1.12	accelerates	S	not specified	ET	Ville and Bissell (1949)
	8.14×10^{-4}	1.32	-	S	Oregon-R	ET	Hinton et al. (1951)
	8.14×10^{-4}	1.12	accelerates	S	Oregon-R	ET	Hinton (1956)
	2.3×10^{-3}	-	accelerates	S	Oregon-S	LT	Sang (1957)
	8.14×10^{-4}	4.67	-	S	Oregon-R	LT	Ville (1950)
Adenosine	6.17×10^{-4}	1.51	-	S	Oregon-R	ET	Hinton et al. (1951)
	6.17×10^{-4}	1.20	accelerates	S	Oregon-R	ET	Hinton (1956)
	2.3×10^{-3}	-	accelerates	S	Oregon-S	LT	Sang (1957)
Deoxyadenosine-----NOT STUDIED-----							
Guanine	5.0×10^{-4}	-	accelerates slightly	Y	Swedish-b	ET	Wilson (1943a)
	7.3×10^{-4}	1.36	-	S	Oregon-R	ET	Hinton et al. (1951)
	7.3×10^{-4}	1.03	accelerates	S	Oregon-R	ET	Hinton (1956)
	2.3×10^{-3}	-	no significant effect	S	Oregon-S	LT	Sang (1957)
	7.3×10^{-4}	7.00	-	S	Oregon-R	LT	Ellis (1950)
Guanosine	5.8×10^{-4}	0.84	-	S	Oregon-R	ET	Hinton (1956)
	5.8×10^{-4}	0.94	accelerates	S	Oregon-R	ET	Hinton (1956)
	2.3×10^{-3}	-	accelerates	S	Oregon-S	LT	Sang (1957)
Deoxyguanosine-----NOT STUDIED-----							

¹Concentrations were converted to Molarity for easier comparison; the highest concentration used by an author is reported.

²Estimated as relative to controls.

³Y: Yeast, S: Basal defined medium (Schultz et al., 1948), S: Sang's defined medium (Sang, 1954), SM: Sang's modified.

⁴ET: Egg transfer, LT: Larval transfer

showed that ^{15}N -adenine can be incorporated into purines of RNA and DNA in the rat. However later studies by Brown *et al.* (1949) established appreciable incorporation of ^{15}N -guanine into mouse nucleic acids. Moreover, when the more easily detectable ^{14}C isotope was used, Abrams (1951) and Balis, Marrian and Brown (1951) showed that guanine and hypoxanthine were used by the rat for RNA synthesis, though to a much lower extent than was reported in the mouse. This fact could be due to the more rapid catabolism of guanine by the highly active enzyme, guanine deaminase, found in the rat tissue (Schulman, 1954). The findings that yeast (Kerr *et al.*, 1951), *Lactobacillus casei* (Balis, Brown, Elion, Hitchings and VenderWerff, 1951; Balis *et al.*, 1952) and the protozoan *Tetrahymena geleii* (Flavin and Graff, 1951) can effectively utilize guanine for the synthesis of RNA purines stress the importance of keeping in mind species differences and that metabolic events in one organism can not be assumed to apply to another organism, Lagerkvist *et al.* (1955) also showed that uracil was readily incorporated into nucleic acids of rapidly growing tissues of the rat, where anabolism is favoured over catabolism, in contrast to the situation in adult tissues. Furthermore, Canellakis (1957) found that even adult tissues can incorporate uracil if it is administered in sufficiently high concentrations to overcome the degradation.

In contrast to the early negative results obtained by the use of free bases (with the exception of adenine), nucleosides seemed from the beginning to be readily incorporated into nucleic acids. Hammarsten *et al.* (1950) showed that the injection of either labelled cytidine or uridine resulted in the incorporation of the compound into both RNA

and DNA of the rat. Similar experiments by Reichard and Estborn (1951) established the incorporation of deoxycytidine and thymidine into the DNA only. ^{15}N -guanosine was found ineffective as a purine precursor for either nucleic acid in the rat (Hammarsten and Reichard, 1950). However, low but significant incorporation was observed when Lowy *et al.* (1952) used guanosine isotopically labelled with ^{14}C . They also established that adenosine and inosine were utilized for the synthesis of RNA purines. However the utilization of adenosine was much lower than that reported by Brown *et al.* (1948) for adenine. Similar results were reported in yeast (Kerr *et al.*, 1951). This could be attributed to rapid deamination of adenosine to inosine by adenosine deaminase (Schulman, 1954) or to the degradation of adenosine to adenine before it could be utilized *per se* for nucleic acid synthesis (Kerr, *et al.*, 1951).

In *Drosophila*, the *in vivo* incorporation of uracil, uridine and thymidine into nucleic acids was demonstrated by Rizki, Douthit and Rizki (1972), Rizki and Rizki (1973), Alonso (1973), Sayles *et al.* (1973) and Carpenter (1974). The incorporation of adenine, guanine, hypoxanthine and their respective nucleosides was studied by McMaster-Kaye and Taylor (1959), Becker (1974a) and Johnson *et al.* (1976). All authors reported the incorporation of these compounds into nucleic acids with the exception of Becker (1974a) who failed to observe the incorporation of hypoxanthine, guanine and guanosine in tissue culture.

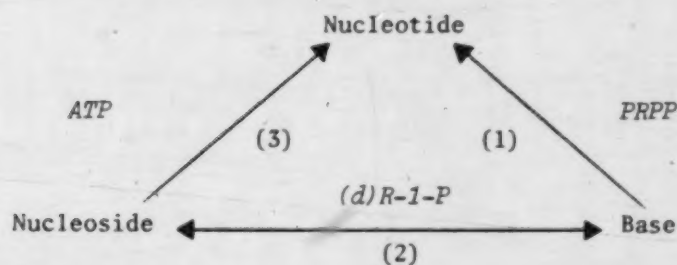
Experiments using labelled compounds on different organisms dominated the field until mid-1950's (for reviews see Christman, 1952; Schulman, 1954; Brown and Roll, 1955). Utilization or non-utilization

of a compound was shown to depend on the organism used, the type of tissue studied and the compound itself.

III. ENZYMATIC CONVERSION OF BASES AND NUCLEOSIDES

The enzymes involved in the utilization of bases and nucleosides have been known for a number of years. However, little is known about their properties and regulation. The isolation of mutants defective in these enzymes has made it possible to construct a more complete picture of these metabolic pathways, as will be shown below.

The following diagram illustrates the main enzymatic reactions by which bases and nucleosides can be anabolized to nucleoside monophosphates.



In general, nucleotides may be formed directly from the base by the action of phosphoribosyltransferases in the presence of PRPP (Reaction 1) or from nucleosides by kinases in the presence of ATP (Reaction 3). Nucleosides may also be cleaved phosphorolytically, by nucleoside phosphorylases (Reaction 2) to yield R-1-P and the base.

Bases may also be converted first to the nucleoside and then to the nucleotide by the sequential action of nucleoside phosphorylases

and kinases respectively (Reactions 2 and 3). It has to be mentioned, however, that although the nucleoside phosphorylase reactions are reversible, they are primarily catabolic (Gots, 1971). The anabolism of bases by these enzymes is limited by the availability of R-1-P and they function only under certain conditions which cause the accumulation of R-1-P. Phosphorolysis, reverse phosphorolysis and kinase activities are also known with respect to the synthesis of deoxy-compounds. Although the above mentioned reactions are the most common ones known from studies on a variety of different species, they are not the only known reactions. Some organisms utilize these compounds in a different manner, as will be discussed below.

Although the above discussion revolves around the interconversion between a single base and its nucleoside and nucleotide derivatives, interconversion between different bases, nucleosides and nucleotides is also important. Such mechanisms enable single compounds to act as sources for a group of related compounds. Figures 1 and 5 summarize the interconversion of pyrimidines and the interconversion of purines, respectively.

The following sections are concerned with the fate of the individual pyrimidine and purine bases and nucleosides, including utilization up to the nucleoside monophosphate level, interconversion and catabolism and with the enzymes involved in these reactions. Interconversion of nucleotides is generally beyond the scope of this review and the reader is referred to Henderson and Paterson (1973) for a review of that topic.

Figure 1. Salvage biosynthesis and interconversion of pyrimidine nucleotides. *De novo* synthesis leads to the production of UMP. (Information derived from Beck *et al.*, 1972a; Henderson and Paterson, 1973).

A. The pyrimidines

The pyrimidine *de novo* biosynthetic pathway is practically universal, with UMP as the final common precursor to the five classes of pyrimidines commonly found in nucleic acids (see Henderson and Paterson, 1973). However, some variation is observed in utilization of preformed pyrimidines, particularly in regard to cytosine and thymine containing compounds.

1. Thymine

Goodman (1974) and Selman and Kafatos (1974) showed that thymine is incorporated at an extremely low rate into animal DNA. Siminovitch and Graham (1955), Crawford (1958) and Bodmer and Grether (1965) failed to show thymine incorporation into DNA of bacteria and it was initially thought that bacteria cannot utilize this base. However, *thy*⁻ mutants, which lack thymidylate synthetase, *can* utilize thymine: hence, enzymes must exist for the conversion of thymine to dTMP.

i. Thymidine phosphorylase [E.C.2.4.2.4]

This enzyme is found in most microorganisms (Razzell and Khorana, 1958, Imada and Igarasi, 1967; Saunders *et al.*, 1969) except in *Lactobacilli* (O'Donovan and Neuhard 1970). It is thought to be located near the cell membrane in *E. coli* (Kammen, 1967; Munch-Petersen, 1967). The distribution of the animal enzyme shows species and tissue differences (Friedken and Roberts, 1954; Krenitsky *et al.*, 1964 and 1965, Zimmerman and Seidenberg, 1964; Weinstock *et al.*, 1973) being absent from some dog tissues (Krenitsky *et al.*, 1965) and Novikoff

hepatoma (Morse and Potter, 1965). The mammalian enzyme shows overlapping specificity with uridine phosphorylase (see below 3. *Uracil*) although they are readily distinguished from one another by DEAE cellulose chromatography (Krenitsky *et al.*, 1964) and their reaction mechanisms (Zimmerman and Seidenberg, 1964; Krenitsky, 1968). Indications of the presence of this enzyme in *Drosophila* were reported by Clynes and Duke (1975).

This enzyme normally cleaves thymidine and deoxyuridine to the base and dR-1-P, as will be discussed later, but in special cases it can anabolize thymine, uracil and certain analogues to their respective deoxyribosides. Kammen (1967), Munch-Petersen (1967), Budman and Pardee (1967), Dale and Greenberg (1972) and Goodman (1974) observed extensive incorporation of this base into DNA of various organisms when deoxyribosides were added simultaneously with thymine, the purine deoxyribosides being the most effective stimulants. Thus it appears that the incorporation of thymine is dependent on the availability of a dR-1-P donor. This suggestion is supported by the finding of Budman and Pardee (1967) and Munch-Petersen (1968a) that deoxyadenosine does not promote thymine incorporation into DNA of mutants defective in either thymidine phosphorylase or purine nucleoside phosphorylase. Furthermore, Breitman and Bradford (1967), Munch-Petersen (1968a) and Lomax and Greenberg (1968) found that mutants defective in enzymes involved in the catabolism of dR-1-P (*i.e.* phosphodeoxyribomutase and deoxyriboaldolase, see Fig. 3) show greater efficiency in utilization of thymine and that *thy*⁻ strains deprived of exogenous thymine release significant amounts of dR-1-P

into the medium.

There are two known mechanisms by which thymidine phosphorylase can transfer the deoxyribosyl moiety to thymine to form thymidine: direct transfer and coupled transfer.

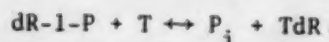
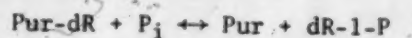
Direct transfer occurs strictly between substrates of thymidine phosphorylase (thymidine, deoxyuridine and analogues) in the following manner:



The enzymes of both *E. coli* and mammalian cells can catalize this reaction. Zimmerman and Seidenberg (1964), Gallo and Breitman (1968) and Krenitsky (1968) reported that dR-1-P is an intermediate in this reaction though it is enzyme bound. In addition, Krenitsky (1968) found that the transfer does not require a stoichiometric amount of phosphate.

Coupled transfer is a phosphate dependent reaction and involves the formation of the free dR-1-P intermediate. The reaction can occur between purine or pyrimidine deoxyribosides and thymine. Hence it requires the joint action of purine nucleoside phosphorylase and thymidine phosphorylase (Gallo and Breitman, 1968) or, in cases where it can cleave deoxyribosides, as in mammals, uridine phosphorylase (Krenitsky *et al.*, 1965).

The reactions are sequential:



The anabolic function of thymidine phosphorylase is the only route by which thymine, *via* the obligate intermediate thymidine, can be metabolized to dTMP in *E. coli*. No phosphoribosyltransferase reaction is known for thymine in this bacterium, since Fangman (1969) showed that upon introduction of thymidine phosphorylase deficiency into a *thy*⁻ strain, the resulting double mutant was a thymidine auxotroph which had lost the ability to survive on thymine. Mutants in this enzyme are unable to cleave deoxyuridine and cannot use it as a sole carbon source (Beck *et al.*, 1972a).

ii. Uridine phosphorylase [E.C.2.4.2.3]

The mammalian uridine phosphorylase can assume the function of thymidine phosphorylase because of their overlapping specificity, or when the latter is absent from specific tissues (see above). Uridine phosphorylase can only anabolize thymine *via* coupled transfer reaction and does not have the capacity for direct transfer (Zimmerman and Seidenberg, 1964; Krenitsky *et al.*, 1965; Krenitsky, 1968; Kraut and Yamada, 1971).

iii. Trans-N-deoxyribosylase [E.C.2.4.2.6]

Many *Lactobacilli* have an absolute growth requirement for a deoxyriboside, a purine and a pyrimidine. This suggests that *Lactobacilli* are capable of synthesizing all their nucleoside triphosphates requirements from such precursors. However, Imada and Igarassi (1967) reported the absence of nucleoside phosphorylases from these bacteria; hence, they must have a different mechanism for transferring deoxyribosyl from one base to another. MacNutt (1952)

discovered that *Lactobacilli* have a specific enzyme, *trans-N*-deoxyribosylase which catalyzes phosphate independent transfer of the deoxyribosyl moiety, without the formation of dR-1-P. Furthermore, the studies of Roush and Bitz (1958), Kanda and Takagi (1959) and Beck and Levin (1963) showed that the synthesis of this enzyme is under repressor control and that cytosine and deoxycytidine are among its substrates. They also indicated that there might be two or more enzymes catalyzing such a deoxyribosyltransfer, one specific for purine-purine transfer. This suggestion was proved recently by Holguin and Cardinaud (1975) using affinity column chromatography.

2. Thymidine

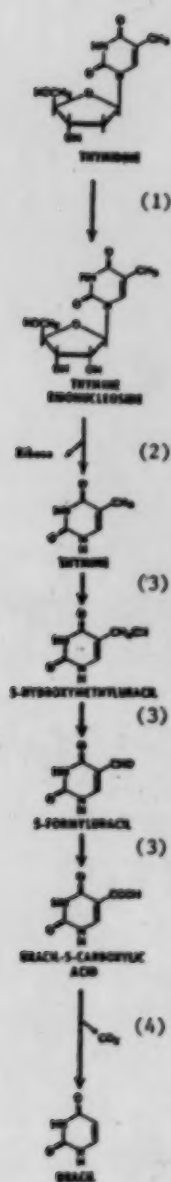
Thymidine is not essential for nucleotide metabolism except in organisms which cannot make the thymine moiety themselves. Nevertheless the utilization of thymidine has received a great deal of attention because of its usefulness in studying DNA synthesis.

In general thymidine, after intracellular phosphorylation to dTMP by thymidine kinase, is readily incorporated into DNA of most cell types (Boyce and Setlow, 1962; Bodmer and Gerther, 1965; Cleaver, 1967). However Adelstein *et al.* (1964) reported that some rodents appear to be severely limited in their ability to utilize thymidine for DNA synthesis, though the limitation was not uniform in all tissues. *Neurospora*, *Aspergillus*, *Saccharomyces* (Grivell and Jackson, 1968) and *Euglena* (Cook, 1966) lack thymidine kinase and do not incorporate thymidine, as such, into their DNA. Fink and Fink (1961, 1962 a and b) found that although ^{14}C -thymidine, labelled

at various positions in the pyrimidine ring, was not incorporated preferentially into *Neurospora crassa* DNA, it labelled the pyrimidine moieties of both RNA and DNA. Mutant studies by Williams and Mitchell (1969) and Schaffer *et al.* (1975) and enzymological studies by Abbott and co-workers (for review see Abbott and Udenfriend, 1973) suggest that this result is due to demethylation of the thymine ring via the pathway shown in Fig. 2. Schaffer *et al.* (1975) suggested that the enzyme pyrimidine deoxyribonucleoside-2-hydrolase catalyzes the major reaction by which *Neurospora* initiates conversion of thymidine to usable pyrimidines. Berry *et al.* (1970) found that the injection of *Cecropia* pupae with ^{14}C -2-thymidine was more effective in labelling thymine moieties of DNA than (methyl- ^{14}C)-thymidine suggesting demethylation at the deoxyriboside level. This was supported by the finding that labelled hydroxymethyl-5-deoxyuridine, but not deoxyuridine, was detected when (methyl- ^{14}C)-thymidine was used and that labelled deoxyuridine was detected only when ^{14}C -2-thymidine was used.

In *Drosophila*, Rizki, Douthit and Rizki (1972); Rizki and Rizki (1973) and Carpenter (1974) found that feeding larvae on (methyl- ^3H)-thymidine resulted in the incorporation of the label into their DNA. This incorporation was shown to increase upon the administration of various analogues which inhibit *de novo* dTMP synthesis. Furthermore the demethylation pathway observed in the silkworm (Berry *et al.*, 1970) seems not to be highly active, if at all, in *Drosophila* since pyrimidine requirements of auxotrophs are not satisfied by thymidine (el Kouni and Nash, unpublished results).

Figure 2. Proposed pathway for the utilization of thymidine by *Neurospora crassa*. The following enzymes mediate the steps as shown: (1) Pyrimidine deoxyribonucleoside-2-hydrolase; (2) Hydrolase; (3) Thymine-7-hydroxylase; (4) Uracil-5-carboxylic acid decarboxylase. (Modified from Schaffer *et al.*, 1975).



Incorporation of thymidine into DNA was observed to cease after a short while in microorganisms (Rachmeler *et al.*, 1961; Bodmer and Grether, 1965), in insects (Selman and Kafatos, 1974) and in plants (Zilberstein *et al.*, 1973a) due to the induction of the enzyme thymidine phosphorylase and subsequent rapid degradation of thymidine to thymine. Conversion of thymidine to thymine in *Drosophila* crude extracts was observed by Clynes and Duke (1975).

i. Thymidine kinase [E.C.2.7.1.75]

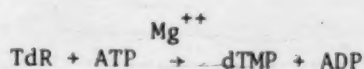
This enzyme is of wide distribution among organisms. However, it is absent from those which cannot incorporate thymidine into their DNA (see above). It is also absent from some mutants selected as resistant to BUdR (Littlefield, 1965, Kit *et al.*, 1963; Freed and Mezger-Freed, 1973) or to FUdR (Morris and Fischer, 1963; Morse and Potter, 1965; Beck *et al.*, 1972a).

Two forms of thymidine kinase are found in vertebrate cells. The principal form of thymidine kinase is found in the cytosol and differs in electrophoretic mobility and other properties from a second form found in the mitochondria (Kit, 1976).

Several workers (see Kit, 1976) have demonstrated that there is a correlation between the activity of the enzyme and the occurrence of DNA synthesis in the cell. It has been documented by various investigators that in a variety of cells the activity of thymidine kinase is altered in response to changes in biological needs. There is an increase of enzyme activity in regenerating mammalian liver (Maley *et al.*, 1965; Bresnick *et al.*, 1970; Adelstein *et al.*, 1971),

in virus infected cells (Hatanaka *et al.*, 1969; Kit *et al.*, 1970), upon resumption of development or as a response to injury in diapausing pupae of silkworm (Brooks and Williams, 1965) and in mammalian tumors (Sneider *et al.*, 1969; Bresnick and Burleson, 1970). Changes in thymidine kinase activity occur also during mitotic cycle of plants (Hotta and Stern, 1963; Wanka *et al.*, 1964; Harland *et al.*, 1973) and animal cells (Kit, 1976), as a result of dietary variation in animals (Beltz, 1962; Adelstein *et al.*, 1971) and in response to hormonal influence in guinea pigs (Masui and Garren, 1971).

The enzyme from *E. coli* was purified and studied by Okazaki and Kornberg (1964 a and b), that from animals by Bresnick and Thompson (1965) and was studied in crude extracts of *Drosophila* by Clynes and Duke (1975). It catalyzes phosphorylation of thymidine in the following manner:



The enzyme has the same substrate specificity irrespective of the tissue of origin. In addition to thymidine, it accepts deoxyuridine and many of its 5-substituted derivatives as substrates. The relative efficiency of each deoxyriboside as a substrate is probably determined by the size of the 5-substituents. They also seem to be phosphorylated by the same active site, since they inhibit the phosphorylation of one another competitively.

Okazaki and Kornberg (1964b) found that the *E. coli* enzyme is under allosteric regulation. It is activated by dCTP, dCDP and to a lesser extent by dATP. It is feedback inhibited by dTTP but not

by dTMP or dTDP.

Bresnick and Karjala (1964) and Taylor *et al.* (1972) found that the mammalian enzyme is similar to that of *E. coli* with regard to inhibition by dTTP. However, they reported that dCTP also inhibits thymidine kinase from adult rat and human tissue, but has virtually no effect on enzymes from tumor or fetal tissues. In insects, Brooks and Williams (1965) showed that thymidine kinase from the oak silkworm, *Antheraea pernyi*, is inhibited by dTTP and to a lesser extent by dCTP, while Berry *et al.* (1970) found the enzyme from *Cecropia* is inhibited by dCDP and high concentration of thymidine. dTTP does not inhibit enzyme activity in crude extracts of the protozoan *Tetrahymena pyriformis* (Sharp *et al.*, 1966).

The enzyme was shown to be composed of more than one subunit. Its activity could be affected by the state of aggregation of these subunits (see Cleaver, 1967). Enzyme from *Drosophila* also is suggested to exist in a variety of active oligomeric forms (Clynes and Duke, 1975).

ii. *Nucleoside phosphotransferase* [E.C.2.7.1.77]

This enzyme is widely distributed in animal and plant tissues as well as in bacteria (Chao, 1976). It resembles the kinase in its action. Thus it phosphorylates thymidine as well as almost all other pyrimidine and purine ribo- or deoxyribosides to their respective nucleoside monophosphate. However it differs from the kinases in that it utilizes low energy phosphate donors for such reactions. The avian enzyme is also inhibited by ATP, the preferred phosphate

donor for thymidine kinase reactions, but it is not inhibited by dTTP as is the kinase (for references see Brunngraber and Chargaff, 1973; Chao, 1976; Kit, 1976).

The enzyme from the bacterium *Erwinia herbicola* was found to be membrane-bound in contrast to that from *E. coli* and carrots (see Chao, 1976).

iii. *Thymidine phosphorylase* [E.C.2.4.2.4]

The distribution, substrate specificity, mechanism and anabolic function of this enzyme have been discussed earlier (see above 1. *Thymine*). Only its role in the catabolism of thymidine and its regulation will be mentioned here.

The induction of this enzyme by thymidine (Rachmeler *et al.*, 1961, Razzell and Casshyap, 1964) results in the rapid degradation of thymidine to thymine and dR-1-P, with the subsequent cessation of thymidine incorporation into DNA of bacteria (Rachmeler *et al.*, 1961), plants (Zilberstein *et al.*, 1973a) and possibly insects (Selman and Kafatos, 1974). Mutational loss of the enzyme in bacteria (Fangman and Novick, 1966; Fangman, 1969) or its inhibition by azacytidine in mammalian cells (Cihak *et al.*, 1976) prevented the rapid degradation of thymidine and enhanced its incorporation into DNA. Kammen (1967) and Yagil and Rosener (1970) found that deoxyadenosine also enhances the incorporation of thymidine into DNA; although deoxyadenosine was found to induce the enzyme, this was offset since deoxyadenosine also provides the resulting thymine with the dR-1-P necessary to reform thymidine (Budman and Pardee, 1967).

The regulation of thymidine phosphorylase was studied extensively in *E. coli* by several workers, most recently by Buxton (1975), Hammer-Jespersen and Munch-Petersen (1975), Albrechtsen *et al.* (1976) and Hammer-Jespersen and Nygaard (1976). The current evidence indicates that the synthesis of thymidine phosphorylase is regulated coordinately with at least three other enzymes, shown in Fig. 3. The four enzymes are coded by four closely linked genes (symbols are shown in Fig. 3) whose linkage relationship is to be found in Fig. 4. All four genes are under the control of two repressor proteins produced by two regulatory genes *deoR* and *cytR*. In the *deoR* system dR-5-P acts as an inducer while in *CytR* system cytidine is the main inducer. However, some evidence suggests that the four genes comprise two transcriptional units: Thus only purine nucleoside phosphorylase and deoxyribomutase are induced by purine nucleosides, whilst induction by cytidine or dR-5-P appeared to lead to production of higher levels of thymidine phosphorylase and aldolase than the other two enzymes; studies with *dra* polar mutations and *deoR* constitutive mutants as well as Mu-insertions apparently confirmed the presence of two transcriptional units (for references see Albrechtsen *et al.*, 1976). More recent genetic experiments carried out on a strain carrying a mutation in *cytR*, along with a *dra* polar mutation, suggest that all four enzymes are cotranscribed, but that a secondary initiation site exists, allowing the transcription of *drm* and *pup* genes independently (Hammer-Jespersen and Munch-Petersen, 1975; Albrechtsen *et al.*, 1976; Hammer-Jespersen and Nygaard, 1976). Fig. 4 illustrates the proposed model for the *deo*-operon(s).

Figure 3. Pathway of thymidine and purine nucleoside catabolism and the enzymes involved. Genes coding for each enzyme are shown in parentheses. (Modified from Buxton, 1975).

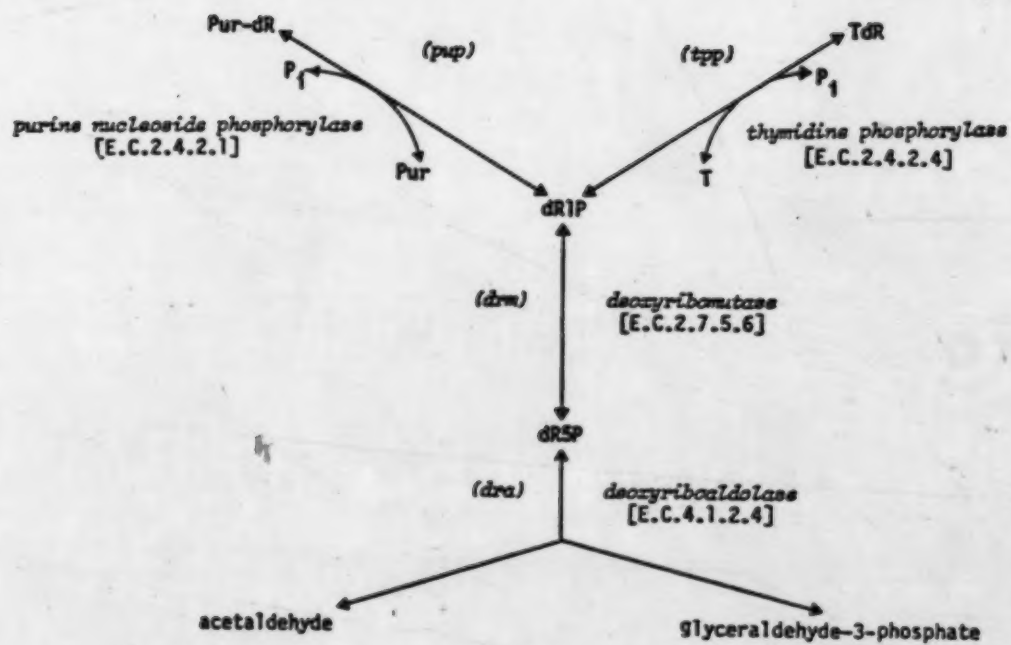
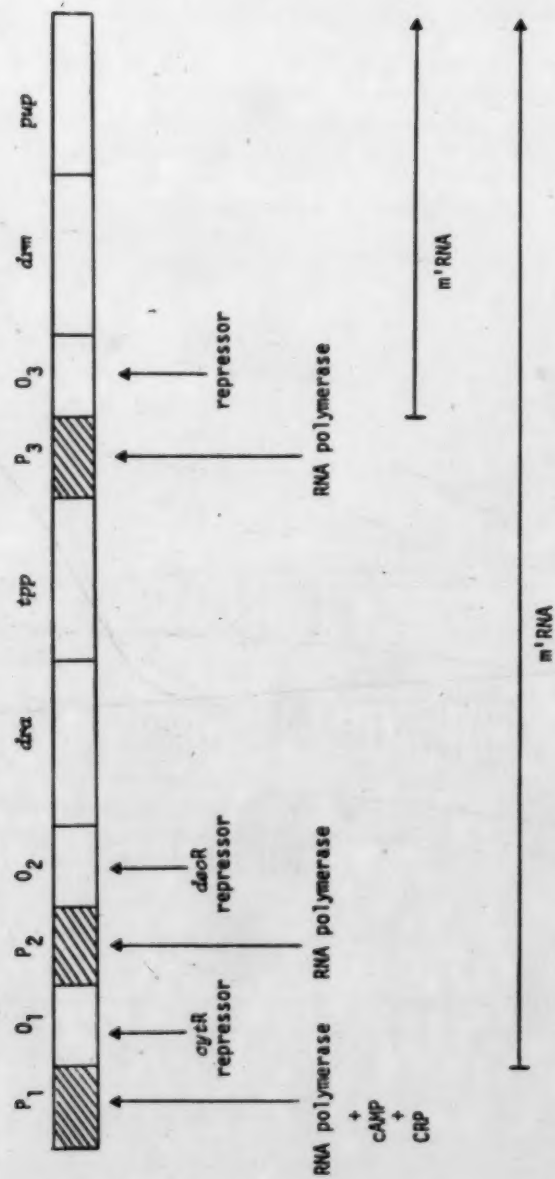




Figure 4. Proposed model for *deo*-operon, P, promotor;
O, operator; cAMP, cyclic AMP; CRP, cAMP receptor
protein. (After Hammer-Jespersen and Nygaard,
1976). For details regarding the control region
to the left of *dra*, see Hammer-Jespersen and
Munch-Petersen (1975).



This model is supported by *in vitro* transcription studies by Svenningsen (1975) using template DNA carrying the four genes. It is the duality of transcriptional origin for the *pup* and *drm* messages and the relatively low amount of the two enzymes coded for by the long transcript (10-30%) that led to the failure of earlier workers to recognize that the four genes constitute a single operon, since mutants and inducers of the *dra* and *tpp* genes affect only the production and translation of the long transcript (Albrechtsen, *et al.*, 1976).

iv. *Uridine phosphorylase* [E.C.2.4.2.3]

The mammalian uridine phosphorylase, though acting preferentially on uridine, can also cleave thymidine and deoxyuridine to thymine and uracil respectively (Krenitsky *et al.*, 1965). Similar activity towards thymidine was reported for the enzyme from *Bacillus stearothermophilus* (Saunders *et al.*, 1969).

v. *Trans-N-deoxyribosylase* [E.C.2.4.2.6]

This enzyme in *Lactobacillus* cleaves thymidine, in the presence of a nucleic acid base, to thymine (see above 1. *Thymine*)

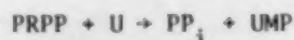
3. *Uracil*

The free base uracil was shown to satisfy the requirements of pyrimidine auxotrophs in bacteria (see O'Donovan and Neuhard, 1970; Beck *et al.*, 1972a) and *Drosophila* (Norby, 1970). However, the uptake of uracil seems to be limited in some animal cells in culture (Hochstadt, 1974).

In general uracil can be anabolized to UMP in one step by uracil phosphoribosyltransferase (UPRT) or in two steps by the sequential action of uridine phosphorylase and uridine-cytidine kinase. It can also be anabolized to dUMP *via* the formation of deoxyuridine by the enzymes thymidine phosphorylase and thymidine kinase.

i. Uracil phosphoribosyltransferase [E.C.2.4.2.10]

In the presence of PRPP, this enzyme anabolizes uracil in the following manner (Reyes, 1969; Reyes and Hall, 1969; Jund and Lacroute, 1972; Reyes and Gubanig, 1975):



This enzyme has been identified in several microorganisms, in *Salmonella typhimurium* (Neuhard, 1968), *E. coli* (Brockman *et al.*, 1960), *Lactobacillus bifidus* (Crawford *et al.*, 1957), *Saccharomyces cerevisiae* and in the protozoan *Tetrahymena pyriformis* (Heinrikson and Goldwasser, 1964). Uracil phosphoribosyltransferase was also found in mammalian cells (Hatfield and Wyngaarden, 1964; Kasbekar *et al.*, 1964; Reyes, 1969; Reyes and Gubanig, 1975) and in *Drosophila* (Clynes and Duke, 1975).

Both the microorganisms and the animal enzymes accept uracil analogues as substrates but not cytosine (Brockman *et al.*, 1960 and Hatfield and Wyngaarden, 1964). However, thymine and orotate were also found to be utilized by the mammalian enzyme, hence the enzyme was called pyrimidine phosphoribosyltransferase (Hatfield and Wyngaarden, 1964 and Reyes and Gubanig, 1975). Nevertheless, the

Drosophila enzyme was shown not to utilize thymine (Clynes and Duke, 1975).

Molloy and Finch (1969) reported that the enzyme from *E. coli* is strongly activated by GTP and inhibited by UMP and UTP.

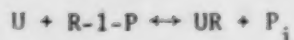
Mutants deficient in this enzyme were selected as either FU or azauracil resistant in bacteria (Brockman *et al.*, 1960; Beck and Ingraham, 1971; Pritchard and Ahmad, 1971; Bean and Tomasz, 1973a), in yeast (Grenson, 1969; Jund and Lacroute, 1970) and in mammalian cells (Kasbekar and Greenberg, 1963). Such bacterial mutants are unable to use uracil but can use uridine as a sole carbon source. Pierard *et al.* (1972) also found that mutation in this enzyme is accompanied by considerable decrease in sensitivity of carbamyl phosphate synthetase [E.C.2.7.2.5] and other enzymes in pyrimidine biosynthesis to repression by uracil. Furthermore, such mutants excrete significant amounts of pyrimidines.

ii. *Uridine phosphorylase* [E.C.2.4.2.3]

This enzyme is found in numerous animals and bacteria (see Henderson and Paterson, 1973). However, it is absent from *Pneumococcus* (Bean and Tomasz, 1972b) and yeast (Jund and Lacroute, 1970). The enzyme was first characterized in bacteria by Paege and Schlenck (1950 and 1952) and in mammalian cell by Krenitsky *et al.* (1964 and 1965).

Although the primary role of this enzyme is the cleavage of uridine to uracil and R-1-P (see 4. *Uridine*), under special

circumstances when dR-1-P is available it can anabolize uracil as follows:



This anabolic function of the enzyme was detected by Koch (1956) in extracts of *E. coli*. The R-1-P is usually provided by the cleavage of a purine nucleoside, hence the transfer is a coupled transfer (see 1. *Thymine*) and dependent on both uridine phosphorylase and purine nucleoside phosphorylase (Imada and Igarassi, 1967). In addition, Krenitsky (1968) demonstrated the exchange of ribose between uridine and ¹⁴C-uracil by uridine phosphorylase. The reaction was phosphate dependent and R-1-P was a free intermediate. Furthermore Pritchard and Ahmad (1971) found that uracil phosphoribosyl-transferase mutants selected as FU or azauracil resistant can be sensitized again to the analogues if adenosine is added to the growth medium as a source of R-1-P. Thus it appears that FU must be anabolized to the toxic FUR by uridine phosphorylase. Under these special conditions (*i.e.* lack of UPRT and availability of R-1-P), Pritchard and Ahmad (*op. cit.*) were able to select for mutants deficient in uridine phosphorylase as FU resistant.

The results of Stroman (1974) suggest the involvement of this enzyme in anabolizing dietary uracil by *Drosophila*.

iii. *Thymidine phosphorylase* [E.C.2.4.2.4]

In the presence of dR-1-P uracil can be converted to deoxyuridine and then to dUMP by thymidine phosphorylase and thymidine kinase

respectively as in the case of thymine (see 1. *Thymine*).

iv. *Trans-N-deoxyribosylase* [E.C.2.4.2.6]

In *Lactobacilli*, this enzyme converts uracil, in the presence of a deoxyribonucleoside, to deoxyuridine (see above 1. *Thymine*).

4. Uridine

Uridine is readily incorporated into nucleic acids of several organisms (see O'Donovan and Neuhard, 1970; Sayles *et al.*, 1973; Hochstadt, 1974). It also satisfies the requirements of pyrimidine auxotrophs in bacteria (O'Donovan and Neuhard, 1970) and *Drosophila* (see for example Falk and Nash, 1974a).

Uridine can be anabolized directly to UMP by the enzyme uridine-cytidine kinase, as most probably is the case in animal cells, or can be cleaved by uridine phosphorylase to uracil which, in turn, can be converted to UMP by uracil phosphoribosyltransferase, as is the case in bacteria (Hochstadt, 1974). Uridine can also be phosphorylated to UMP by nucleoside phosphotransferase (see 2. *Thymidine*).

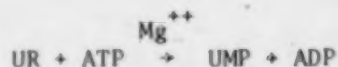
i. *Uridine-cytidine kinase* [E.C.2.7.1.8]

Uridine-cytidine kinase is the only pyrimidine ribonucleoside kinase known (Beck *et al.*, 1972a; Henderson and Paterson, 1973).

The enzyme has been studied in mammalian cells by Sköld (1960), Anderson and Brockman (1964) and Orengo (1969) in sea urchins by Orengo (1966), in bacteria by Anderson and Brockman (1964) and Beck *et al.* (1972a) and in crude extracts of *Drosophila* by Clynes and

Duke (1975).

The enzyme phosphorylates uridine in the following manner (Sköld, 1960):



Although uridine, cytidine and several of their analogues seem to be substrates for this enzyme (Sköld, 1960; Cihak *et al.*, 1964; O'Donovan and Neuhard, 1970), orotidine and pyrimidine deoxyribosides are not (Sköld, 1960; Cihak and Vesely, 1973). Anderson and Brockman (1964) and Orengo (1969) have also shown that bacterial and mammalian enzymes are inhibited by CTP and UTP and stimulated by ATP and dGTP. Evidence for such a control system *in vivo* has been obtained by Neuhard (1968) in mutants of *S. typhimurium*.

Mutants deficient in this enzyme were isolated as FUR resistant in yeast (Grenson, 1969) and *Pneumococci* (Bean and Tomasz, 1973a) and resistant to both FUR and FCR in *S. typhimurium* (Neuhard, 1968; Beck *et al.*, 1972a), thus suggesting that uridine and cytidine are phosphorylated by the same kinase.

Stroman (1974) suggested the absence of this enzyme in *Drosophila* mutants withered (*whd*) and tilt (*tt*).

ii. Nucleoside phosphotransferase [E.C.2.7.1.77]

The enzyme phosphorylates uridine to UMP using low energy phosphate donors [see 2. Thymidine].

iii. *Uridine phosphorylase* [E.C.2.4.2.3]

The distribution of this enzyme, its mechanism and its ability to convert uracil to uridine are mentioned above (see 3. *Uracil*). In spite of the anabolic function of uridine phosphorylase, its main rôle is certainly catabolic. Beck and Ingraham (1971) and Beck *et al.* (1972a) reported that the enzyme functions only catabolically *in vivo*; strains of *S. typhimurium* deficient in uracil phosphoribosyltransferase were shown to be unable to utilize uracil and completely resistant to FU. Furthermore Beck *et al.* (1972a) noticed that 75% of dietary uridine was cleaved to uracil and concluded that, *in vivo*, the major route for utilization of uridine is *via* its cleavage to uracil by phosphorolysis.

Partially purified enzymes from *E. coli* and *S. typhimurium* were shown to cleave uridine and certain analogues (Cihak *et al.*, 1964) but not cytidine, orotidine or deoxyuridine (Paegle and Schlenck, 1952; Razzell and Khorana, 1958 and Beck and Ingraham, 1971). Saunders *et al.* (1969) found that the enzyme from *B. stearothermophilus* cleaves thymidine also. Krenitsky *et al.* (1965) reported that the mammalian enzyme can cleave deoxyuridine, FUDR and thymidine, though less actively than uridine, the dog enzyme being exceptional for its preference for the deoxyribosides.

Krenitsky *et al.* (1965) found that there are two classes of vertebrate uridine phosphorylase enzymes, depending upon their pH optima. One has an optimum pH of about 6.5 (Chick, human, guinea pig and frog) and the other of about 8 (mouse, rat and dog).

Uridine phosphorylase may also be found in *Drosophila*; in addition to the report of Stroman (1974) mentioned above (see 3. *Uracil*, *ii.*). Clynes and Duke (1975) reported that the conversion of uridine to uracil by *Drosophila* extracts.

Mutants in this enzyme were selected in bacteria by Neuhard and Ingraham (1968) and Pritchard and Ahmad (1970) on the basis of inability to grow on uridine as a sole carbon source.

It is interesting to note that although the structural gene for uridine phosphorylase, in *E. coli* and *S. typhimurium*, is not linked to that of thymidine phosphorylase, both are induced by cytidine and negatively controlled by the same repressor protein coded for by *cytR* in the *deo* operon (for references see Hammer-Jespersen and Nygaard, 1976 and Fig. 4).

iv. Nucleoside ribohydrolase [E.C.3.2.2.3]

Although uridine is generally cleaved phosphorolytically in most organisms, as mentioned above, it is cleaved hydrolytically in *Saccharomyces cerevisiae* (Carter, 1951) and *Lactobacillus pentosus* (Wang and Lampen, 1951; Lampen and Wang, 1952) by the enzyme nucleoside ribohydrolase. These enzymes have relatively little specificity with regard to their riboside substrates. Purine as well as pyrimidine ribosides can be cleaved by such enzymes. A mutant of this enzyme, unable to cleave uridine, has been reported by Grenson (1969) in yeast.

5. Deoxyuridine

Deoxyuridine serves as a total source of carbon and energy in bacteria (Beck *et al.*, 1972a). It also satisfies the requirements of bacterial pyrimidine auxotrophs.

Deoxyuridine can either be anabolized to dUMP by thymidine kinase or nucleoside phosphotransferase, or catabolized to uracil by thymidine or uridine phosphorylase in most organisms and by *trans*-N-deoxyribosylase in *Lactobacilli* (for discussion of these enzymes see 1. *Thymine* and 2. *Thymidine*). However, Bean and Tomasz (1973b) reported the absence of any enzymes which cleave the N-glycosidic bonds of pyrimidine deoxyribosides in *Pneumococcus*.

6. Cytosine

Mammalian cells are unable to utilize cytosine (Hochstadt, 1974). In *Drosophila* Norby (1970) showed that cytosine cannot satisfy the nutritional requirement of *rudimentary* mutants, which are pyrimidine auxotrophs. Most microorganisms can utilize this base only *via* its deamination to uracil by the enzyme cytosine deaminase. This was illustrated by the findings of Beck *et al.* (1972a) that the pyrimidine requirements of *S. typhimurium* pyrimidine auxotrophs which are also deficient in cytosine deaminase are satisfied by uracil but not by cytosine. Furthermore, temperature sensitive uracil phosphoribosyltransferase mutants were shown to be fluorocytosine (FC) resistant at the restrictive temperature. These results indicate the absence of any nucleoside phosphorylase or phosphoribosyltransferase activities for cytosine. This was further confirmed by Neuhaard and Ingraham

(1968) when they demonstrated that cytosine cannot support growth of cytidine requiring mutants (lacking cytidine deaminase and CTP synthetase, see Fig. 1). However cytosine can be anabolized to deoxycytidine in *Lactobacilli* by *trans-N*-deoxyribosylase (see 1. *Thymine*).

i. Cytosine deaminase [E.C.3.5.4.1]

Beck *et al.* (1972a) reported that the deamination of cytosine by this enzyme is the only route by which this base can be utilized in *S. typhimurium*. The enzyme is active enough to meet the entire growth requirements for nitrogen in *E. coli* (Beck *et al.*, 1972a) and yeast (Grenson, 1969). It is also suggested that deamination is the rate-limiting step in the utilization of cytosine (Bean and Tomasz, 1973b).

Cytosine deaminase is found in several microorganisms (O'Donovan and Neuhard, 1970) but not in animal cells (see Henderson and Paterson, 1973). Kream and Chargaff (1952) and Hayaishi and Kornberg (1952) reported that the enzyme deaminates cytosine to uracil in the following manner:



The enzyme is also capable of deaminating the cytosine analogues; 6-azacytosine, 5-fluorocytosine and isocytosine (O'Donovan and Neuhard, 1970). However Cohen (1953) found that the *E. coli* enzyme is unable to deaminate 5-methylcytosine, a reaction which has been demonstrated earlier by Kream and Chargaff (1952) in yeast. The *E. coli* enzyme is also inhibited by 5-azacytosine and 5-azauracil (Cihak and Sorm, 1965).

Mutants deficient in this enzyme were isolated in prototrophic *S. typhimurium* by Neuhard (1968) and Beck *et al.* (1972 a and b) and in *E. coli* by Ahmad and Pritchard (1972), as mutants resistant to FC but not to FU, and in pyrimidine auxotrophs in *S. typhimurium* as strains unable to use cytosine as a sole pyrimidine source (Beck *et al.*, 1972b). Mutants were also isolated in yeast by Grenson (1969) and Jund and Lacronte (1970).

ii. Trans-N-deoxyribosylase [E.C.2.4.2.6]

In *Lactobacilli*, this enzyme will convert cytosine to deoxycytidine provided that a deoxynucleoside is also available for the reaction (see above 1. *Thymine*).

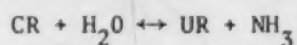
7. Cytidine

Cytidine can be utilized by both mammalian cells and microorganisms. It can be phosphorylated to CMP by uridine-cytidine kinase or deaminated to uridine by cytidine deaminase. Mutants defective in both of these enzymes are unable to utilize cytidine, demonstrating the absence of cytidine phosphorylase activity.

i. Cytidine deaminase [E.C.3.5.4.5]

This enzyme is found in various animal cells (see Henderson and Paterson, 1973) and in *S. typhimurium* and *E. coli* (Wang *et al.*, 1950). The *E. coli* enzyme is located in the membrane (Munch-Peterson, 1968b). It is absent from *Lactobacilli* (Wang and Lampen, 1951) and yeast (Grenson, 1969).

The enzymes from *E. coli*, *S. typhimurium* and sheep liver were studied by Wang *et al.* (1950); Cohen and Barner (1957), Beck *et al.*, (1972a) and Wisdom and Orsi (1969). The enzyme deaminates cytidine (as well as deoxycytidine, their 5-halogenated derivatives and 5-methyldeoxycytidine) in the following manner:



This reaction is very rapid in *E. coli* and *S. typhimurium* (Beck *et al.*, 1972a) so that it provides sufficient NH_3 to meet total nitrogen requirements (O'Donovan and Neuhard, 1970; Beck *et al.*, 1972a). By reason of rapid deamination, cytidine requiring mutants (lacking CTP synthetase, see Fig. 1) can only be selected in a genetic background of cytidine deaminase deficiency (Neuhard and Ingraham, 1968; Beck and Ingraham, 1971). Cohen and Barner (1957) showed that deamination of 5-methyldeoxycytidine by this enzyme fulfils the thymidine requirements of *thy*⁻ mutants in *E. coli*.

Cytidine deaminase activity correlates well with the presence or absence of active development in *Cecropia* (Berry and Firshein, 1967; Firshein *et al.*, 1967).

The synthesis of cytidine deaminase in bacteria is induced by high concentrations of cytidine as is the case for thymidine phosphorylase and uridine phosphorylase. The structural genes for all three enzymes, although unlinked, are negatively controlled by *cytR* gene in the *deo* operon (for references see Hammer-Jespersen and Nygaard, 1976 and Fig. 4). Thus, it appears that the phosphorolysis of all pyrimidine ribo- and deoxyribonucleosides in bacteria is controlled by a

common regulatory gene (i.e. *cytR*).

Cytidine deaminase mutants were isolated in *E. coli* (Karlstrom, 1968), *S. typhimurium* (Neuhard and Ingraham, 1968; Neuhard, 1968; Beck and Ingraham, 1971; Beck *et al.*, 1972a) and in *Pneumococcus* (Bean and Tomasz, 1973a) as strains resistant to FCdR but sensitive to FUDR or as strains unable to use deoxycytidine either as a sole nitrogen source (Neuhard and Ingraham, 1968) or as a sole pyrimidine source (Karlstrom, 1968).

ii. *Uridine-Cytidine kinase* [E.C.2.7.1.8]

This enzyme is responsible for the utilization of cytidine and toxicity of FCR in cytidine deaminase mutants in bacteria (Neuhard and Ingraham, 1968). The distribution, properties and mutants of this enzyme have been discussed above (see 4. *Uridine*).

iii. *Nucleoside phosphotransferase* [E.C.2.7.1.77]

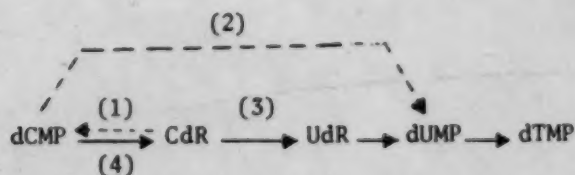
This enzyme can anabolize cytidine to CMP using low energy phosphate donors (see 2. *Thymidine*).

iv. *Nucleoside ribohydrolase* [E.C.3.2.2.3]

Although, as mentioned above, *Lactobacilli* and yeast are devoid of cytidine deaminase, these species can cleave cytidine hydrolytically by the enzyme nucleoside ribohydrolase (see 4. *Uridine*).

8. Deoxycytidine

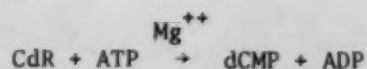
The utilization of deoxycytidine seems to differ according to the organism under investigation. Mammalian cells were shown to be capable of forming dCMP from deoxycytidine by the enzyme deoxycytidine kinase (Durham and Ives, 1970 and Ives and Durham, 1970). They are also capable of deaminating deoxycytidine to deoxyuridine by the enzyme cytidine deaminase (see 7. *Cytidine*). On the other hand bacteria, with the exception of *Lactobacilli*, utilize deoxycytidine only *via* deamination. This was demonstrated by the finding of Beck *et al.* (1972a) that pyrimidine auxotrophs that are also defective in cytidine deaminase (see Fig. 1) are able to grow on deoxyuridine but not on deoxycytidine as a sole pyrimidine source; hence they suggested the absence of any deoxycytidine kinase or phosphorylase in *S. typhimurium*. A similar conclusion was reached by Firshein *et al.* (1967), studying homogenates of developing *Cecropia* pupae injected with ^{14}C -deoxycytidine, in which they observed extensive activity of cytidine deaminase. In fact, the work of Berry and Firshein (1967), Firshein *et al.* (1967), Berry *et al.* (1970) and Swindlehurst *et al.* (1971) suggests that, in *Cecropia*, the principal fate of exogenous deoxycytidine is in thymine nucleotides. Their proposed pathway for such synthesis is summarized in the following diagram:



Appropriate intermediates involved in this pathway were identified when labelled deoxycytidine or deoxyuridine were incubated with extracts of *Cecropia*. Other evidence supports this suggestion. It was shown that injected deoxyuridine or deoxycytidine labelled only thymidine moieties of DNA. In addition, no detectable activities of deoxycytidine kinase (1), dCMP deaminase (2) or any deoxycytidine degradative enzyme were found, while high activities were reported for cytidine deaminase (3) and dCMP nucleotidase (4). Also, the omission of THFA from reaction mixture containing deoxyuridine or deoxycytidine resulted in no detectable dTMP.

i. Deoxycytidine kinase [E.C.2.7.1.74]

This enzyme has been found in animal tissues (Henderson and Paterson, 1973). However, it was not detected in *Cecropia* (Berry and Firshein, 1967; Firshein *et al.*, 1967). It is absent from *S. typhimurium* (Neuhard, 1968) and *E. coli* (Karlstrom, 1970) but present in *Lacotobacillus acidophilus* (Durham and Ives, 1971). The enzyme was purified from *L. acidophilus* (Durham and Ives, 1971) and calf thymus (Krenitsky *et al.*, 1976). The enzyme phosphorylates deoxycytidine in the following manner:



The phosphate can be provided by any ribo- or deoxyribonucleoside triphosphate except dCTP, which is an allosteric inhibitor of the enzyme. Such inhibition was found to be reversed by dTTP. Deoxycytidine kinase is relatively specific for the pentose rather than the base moiety. The enzyme can phosphorylate deoxyadenosine and

deoxyguanosine, though less effectively than deoxycytidine. Cytidine, uridine and thymidine are not substrates for this enzyme.

ii. *Nucleoside phosphotransferase* [E.C.2.7.1.77]

This enzyme can anabolize deoxycytidine to dCMP (see 2. *Thymidine*).

iii. *Cytidine deaminase* [E.C.3.5.4.5]

The characteristics and role of this enzyme in anabolizing deoxycytidine and cytidine have been discussed above (see 1. *Cytidine*).

iv. *Trans-N-deoxyribosylase* [E.C.2.4.2.6]

This enzyme from some *Lactobacilli* transfers the deoxyribose moiety from deoxycytidine to a pyrimidine or a purine base to yield cytosine and a deoxynucleoside (see above 1. *Thymine*).

B. The purines

Utilization of purine bases, nucleosides and deoxynucleosides is similar to those discussed for pyrimidine compounds. However instances of anabolic roles for the purine nucleoside phosphorylase and kinases are much less common than those reported for the pyrimidines. Hence the major route for anabolizing purine compounds is *via* phosphoribosyltransferases. The interconversions of purine bases, nucleosides and nucleotides are shown in Fig. 5.

Although the *de novo* synthesis of GMP and AMP and their anabolism to nucleoside triphosphates are practically universal, the

Figure 5. Salvage biosynthesis and interconversion of
purine nucleotides. (Information derived from
Henderson and Paterson, 1973).

conversion of AMP to GMP or *vice versa* seems to differ from one organism to another. For example animals are generally able only to convert AMP to GMP while *Lactobacillus leichmanii* and *Tetrahymena geleii* are able only to convert GMP to AMP. *E. coli* is capable of conversion in either direction (for discussion and references see Naguib, 1976). The utilization of bases, ribosides and deoxyribosides also varies in different organisms (see below).

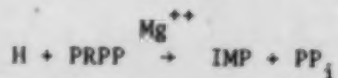
1. Hypoxanthine

Hypoxanthine was shown to satisfy the purine requirement of purine auxotrophic bacteria (Hoffmeyer and Neuhard, 1971). It is mainly used *via* anabolism to AMP by hypoxanthine phosphoribosyltransferase (HPRT) or oxidized to xanthine by xanthine oxidase or xanthine dehydrogenase. In some cases it can react with purine nucleoside phosphorylase to form inosine or deoxyinosine. In some *Lactobocilli* it can be converted to deoxyinosine by *trans*-N-deoxyribosylase

i. Hypoxanthine phosphoribosyltransferase [E.C.2.4.2.8]

The availability of a great number of mutants in this enzyme makes it one of the best studied enzymes in the salvage pathway (Hochstadt, 1974). The characteristics, distribution and physiological role of HPRT were dealt with in a number of reviews, most recently by Murray *et al.* (1970), Murray (1971), Gots (1971) Henderson and Paterson (1973) and Hochstadt (1974).

HPRT catalyzes the following reaction:



The substrate specificity of HPRT varies according to the organism investigated. The mammalian and yeast enzymes catalyze the conversion of hypoxanthine, guanine, and several of their analogues to their respective nucleotides, hence are called hypoxanthine-guanine phosphoribosyltransferase (HGPRT). The animal enzyme reacts weakly with xanthine. In bacteria, a distinct enzyme exists for each base, though cross-specificity with substrate still exists. For example, HPRT from *S. typhimurium* converts 10% of available guanine to GMP, but no xanthine (Benson and Gots, 1975). The evidence for substrate specificity of these enzymes is based on kinetic properties with different substrates and the utilization of various bases and their analogues by different mutant strains. Dissimilarity in electrophoretic mobility between the different enzymes was also observed.

The enzymes (HGPRT, HPRT and GPRT) require PRPP for reaction and are feed-back inhibited by the corresponding nucleotides of the substrates. Such inhibition is competitive with PRPP. Enzyme production is derepressed when cells are dependent on exogenous purines.

Mutants in these enzymes have been selected as resistant to 6-mercaptopurine, 8-azaguanine and 6-thioguanine. These mutants lack or have partial enzyme activity. Mutants completely lacking enzyme activity were found either to have normal levels of crossreacting material (CRM) or to be deficient in CRM (Upchurch *et al.*, 1975). Revertants of HGPRT⁻ mutants are recovered on HAT (hypoxanthine-amethopterin-thymidine) medium (Littlefield, 1964).

Complete absence of enzyme activity (Lesch-Nyhan; Seegmiller *et al.*, 1967) or partial loss of activity (gout; Kelley *et al.*, 1967) is associated with elevation in PRPP-synthetase [E.C.2.7.6.1] activity and PRPP production; hence the increase in *de novo* purine biosynthesis and purine excretion (Becker *et al.*, 1975; Martin and Maler, 1976). This led Martin and Maler (1976) to propose that the HGPRT gene has two functions; one is responsible for HGPRT synthesis and the other function is to regulate PRPP-synthetase synthesis. The loss of the latter function is suggested to be the cause for the increased *de novo* purine biosynthesis and purine excretion, resulting from elevation in PRPP synthesis. This contradicts the earlier belief that the increased PRPP pool in HGPRT mutants is due to the lack of utilization by the defective enzyme.

Arnold and Kelley (1971) distinguished three variants of purified human HGPRT by isoelectric focusing. However, all three variants were shown to have essentially the same substrate specificity and sensitivity to feed-back inhibition by GMP. They suggested that non-genetic post-transcriptional modification, of one or both subunits of the enzyme, is the cause for the observed electrophoretic heterogeneity. Genetic variability was ruled out since the gene for HGPRT is sex-linked and the sample was taken from one male donor and a single mutational event results in total absence of the enzyme. The total lack of enzyme activity for some HGPRT mutants also suggests that polymerization of identical monomers could be responsible for these electrophoretic variants or that these variants share a common subunit (see Hochstadt, 1974).

Complementation studies on HAT medium suggest that there are at least three cistrons in the HGPRT gene in mouse (Sekiguchi *et al.*, 1975).

Hochstadt (1974) implicated HGPRT in the uptake of purine bases in bacteria, since uptake was proportional to enzyme activity. However, Beaudet *et al.* (1973) and Morrow *et al.* (1973) reported that animal cells mutant in hypoxanthine transport or HGPRT activity are azaguanine resistant. In the case of the transport mutants, HGPRT activity was not necessarily absent, suggesting that HGPRT and the hypoxanthine transport system are different entities.

The enzyme seems to be absent from *Musca domestica* (Miller and Collins, 1973) and *Drosophila* (Becker, 1974 a and b). Both species did not incorporate hypoxanthine and are naturally resistant to 8-azaguanine, 6-mercaptopurine and other analogues. No enzyme activity was detected in *Drosophila* extracts (Becker, *op. cit.*). On the other hand, Johnson *et al.* (1976) reported the incorporation of dietary ¹⁴C-hypoxanthine into nucleic acids of *Drosophila*.

ii. *Purine nucleoside phosphorylase* [E.C.2.4.2.1]

This enzyme was identified in many cell types (see Murray *et al.*, 1971; Hochstadt, 1974). The bacterial enzyme seems to be located in the cell membrane (Hochstadt, 1974). Its recognized function *in vivo* is the cleavage of the glycosidic bond of all purine nucleosides, deoxyribonucleosides and their 6-oxypurine analogues, (Friedkin and Kalckar, 1961; Robertson and Hoffee, 1973; Jensen and Nygaard, 1975; Lewis and Glantz, 1976). However, xanthosine is not cleaved in *E. coli*

and *S. typhimurium* (Jensen and Nygaard, 1975). The enzyme acts in the following manner:



Although the reaction is reversible and favours nucleoside synthesis *in vitro* (see Murray *et al.*, 1970) few studies reported such activity *in vivo* (see below). This could be due to the limited availability of (d)R-1-P inside the cell under normal conditions (Hoffmeyer and Neuhard, 1971).

Studies *in vitro* showed that the enzyme catalyzes phosphate dependent "coupled transfer" (see 1. *Thymine*) in purine-purine (deoxy) ribosyl transfer (Krenitsky, 1967) and joins thymidine phosphorylase in purine-pyrimidine deoxyribosyl transfer (Gallo and Breitman, 1968). Furthermore, animal enzymes catalyze a slow, phosphate independent, "direct transfer" reaction (see Murray *et al.*, 1970).

In vivo anabolism of hypoxanthine by purine nucleoside phosphorylase was reported by Raivio and Seegmiller (1973) studying fibroblasts from patients lacking HGPRT. Becker (1974b) failed to detect any anabolic activity of this enzyme towards hypoxanthine in extracts of *Drosophila*. However Hodge and Glassman (1967a) demonstrated the formation of inosine by extracts of *Drosophila* mutants deficient in xanthine dehydrogenase.

Several studies have shown that the structural gene for purine nucleoside phosphorylase, in *E. coli* and *S. typhimurium*, is located within the *deo* operon (for references see Hammer-Jespersen and Nygaard,

1976 and Fig. 4). Although the synthesis of this enzyme could be induced by purine nucleosides, it is also induced by cytidine as is the case for the synthesis of thymidine phosphorylase, uridine phosphorylase, and cytidine deaminase (for references see Hammer-Jespersen and Munch-Petersen, 1975; Hammer-Jespersen and Nygaard, 1976). Thus it appears that the catabolism of all purine and pyrimidine nucleosides is negatively controlled by a common regulatory gene, *cytR*.

Purine nucleoside phosphorylase mutants in humans are associated with deficiencies in the immune system (for references see Ullman *et al.*, 1976).

iii. *Trans-N-deoxyribosylase* [E.C.2.4.2.6]

In some *Lactobacilli*, this enzyme, in the presence of a deoxyribonucleoside, will convert hypoxanthine to deoxyinosine (see 1. *Thymine*).

iv. *Xanthine dehydrogenase* [E.C.1.2.1.37] and
xanthine oxidase [E.C.1.2.3.2]

These two enzymes catalyze the oxidation of hypoxanthine to xanthine. They also oxidize xanthine and other purine and non-purine compounds and both are inhibited by allopurinol. Nevertheless, the dehydrogenases, as in mammals and *Drosophila*, require NAD^+ as an electron acceptor for the reaction, while the oxidases of birds and bacteria use O_2 as an electron acceptor. It is a matter of dispute whether *in vivo* oxidation is carried out by the dehydrogenases and that the oxidases are just a form of the dehydrogenases appearing only upon isolation of the latter. For references and more details

see Henderson and Paterson (1973).

The enzyme from *Drosophila* has received a great deal of attention biochemically and genetically (see review by MacIntyre and O'Brien, 1976) because of its role in eye pigmentation and its utility in studying genetic fine structure and, possibly, genetic regulation in this organism (see Chovnick *et al.*, 1976). The enzyme is a dimer coded for by a structural gene (*rosy*, *ry*) and influenced by at least three other genes, namely *cinnamon* (*cin*), *low xanthine dehydrogenase* (*lxd*) and *maroon-like* (*ma-l*). Mutants lacking this enzyme die on diets supplemented with purine (Glassman, 1965). Barrett and Davidson (1975) explained the non-autonomy of the *rosy*⁺ gene with respect to eye color on the basis that the enzyme is being synthesized outside the eye and then transported to the eye of the pupae *via* the haemolymph. The inhibition of hypoxanthine oxidation by allopurinol was confirmed in *Drosophila* by Johnson *et al.* (1976).

It is noteworthy that Becker (1974b) was able to detect enzyme activity in whole larvae or adult extracts but not from cell cultures. These cell cultures excreted hypoxanthine and xanthine.

2. Inosine

Purine auxotrophs in bacteria (Zimmerman and Magasanik, 1964; Hoffmeyer and Neuhard, 1971) and *Drosophila* (Naguib, 1976) were shown to grow on inosine. It was also shown that dietary ¹⁴C-inosine is incorporated into nucleic acids of *Drosophila* larvae (Johnson *et al.*, 1976).

Inosine can be phosphorylated to IMP by inosine kinase or cleaved to hypoxanthine by purine nucleoside phosphorylase.

i. *Inosine kinase* [E.C. none]

The evidence for the existence of this enzyme is limited. Pierre *et al.* (1967) and Pierre and Le Page (1968) found that crude extracts from HGPRT deficient Ehrlich ascites tumor cells are able to convert inosine to IMP. Furthermore Zimmerman and Magasanik (1964) and Hoffmeyer and Neuhard (1971) showed that bacterial purine auxotrophs also lacking purine nucleoside phosphorylase or HPRT are able to grow on inosine. This route of utilization seems limited since growth on inosine is suboptimal.

In *Drosophila*, Johnson *et al.* (1976) found that 51% of ^{14}C -inosine radioactivity was incorporated into nucleotides while only 4% was catabolized, which suggests, but does not prove, the presence of inosine kinase. The enzyme from *E. coli* phosphorylates both guanosine and inosine. A mutant in this enzyme was isolated by Jochimsen *et al.* (1975).

ii. *Purine nucleoside phosphorylase* [E.C. 2.4.2.1]

This enzyme cleaves inosine to hypoxanthine and R-1-P (see 1. *Hypoxanthine* for general characteristics of the enzyme).

Bacterial mutants in this enzyme show very low survival on inosine but do not survive on hypoxanthine suggesting that this enzyme is the major route for utilization of inosine in bacteria (Hoffmeyer and Neuhard, 1971). Very low activity of this enzyme towards inosine

was detected in *Drosophila* extracts (Becker, 1974b). Hodge and Glassman (1967b) noted the conversion of inosine and deoxyinosine to hypoxanthine in extracts of different strains of *Drosophila*. Mutants affecting xanthine dehydrogenase activity (*ry* and *ma-l*) showed, respectively, 78 and 86% increase in activity of the enzyme towards inosine but not towards deoxyinosine. The base level of activity towards both nucleosides was the same in "Canton-S" wild type.

iii. *Nucleoside ribohydrolase* [E.C.3.2.2.3]

In *bacteria*, this enzyme cleaves inosine, hydrolytically, to hypoxanthine (see 4. *Uridine*).

3. Adenine

This base was shown to satisfy the purine requirements of auxotrophs in bacteria (Zimmerman and Magasanick, 1964; Hoffmeyer and Neuhard, 1971; Jochimsen *et al.*, 1975), yeast (see Heslot, 1972; Plischke *et al.*, 1976), *Neurospora* (Pendyala and Wellman, 1975) and mammalian cells (Patterson *et al.*, 1974). It was shown to be incorporated into nucleic acids of *Drosophila* larvae fed on ¹⁴C-adenine (MacMaster-Kay and Taylor, 1959) and that of cultured cells (Becker, 1974a). Similar results were reported in *Musca domestica* (Miller and Collins, 1973).

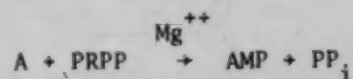
Adenine can be anabolized to AMP by adenine phosphoribosyltransferase or deaminated to hypoxanthine in some bacterial and plant cells. It can also be anabolized to the (deoxy)nucleosides by purine nucleoside phosphorylase in most organisms and to deoxyadenosine by

trans-N-deoxyribosylase in some *Lactobacilli*.

i. *Adenine phosphoribosyltransferase* [E.C.2.4.2.7]

The enzyme (APRT) is found in many organisms and seems to be the main route of utilizing exogenous adenine (Hochstadt, 1974). Raivio and Seegmiller (1973) reported that, in fibroblasts, 75% of the total radioactivity of available ^{14}C -adenine was found in adenine nucleotides even when the conversion of IMP to AMP is blocked by hadacidin. Becker (1974a) reported that *Drosophila* cultured cells deficient in APRT can no longer incorporate ^{14}C -adenine into their nucleic acid, suggesting this to be the only route for adenine utilization.

The enzyme catalyzes the direct conversion of adenine to AMP in the following manner:



APRT is highly specific to adenine, hence the difficulty in using some adenine analogues as substrates (Krenitsky *et al.*, 1969; Gadd and Henderson, 1970). The enzyme has a great competitive advantage for PRPP. This could explain the scarcity of free adenine in some tissues (Henderson and Paterson, 1973). The enzyme is feedback inhibited by ATP and various nucleoside monophosphates (Gots, 1971). Studies with bacteria (see Hochstadt, 1974) and *Drosophila* (Becker, 1974a) implicate the enzyme in adenine transport. The uptake of adenine, in bacteria, was correlated with the activity of this membrane-bound enzyme (see Hochstadt, 1974). Becker (1974a) reported that no labelled adenine was detected in cultured *Drosophila* cells deficient in APRT.

Murray (1967) observed changes in APRT properties of developing mouse embryos and suggested the existence of fetal and adult forms of the enzyme.

Mutants in APRT were obtained in *S. typhimurium* by Kalle and Gots (1963) and Adye and Gots (1966) and were shown to excrete high amounts of adenine. In *Drosophila*, APRT mutants were isolated in cell cultures by Becker (1974a) as azaadenine and fluoroadenine resistant. Autosomally inherited hypoactive and hyperactive variants have been reported in man (Kelley *et al.*, 1968; Henderson *et al.*, 1969).

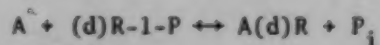
ii. *Trans-N-deoxyribosylase*

In *Lactobacilli*, in the presence of a deoxynucleoside, the enzyme converts adenine to deoxyadenosine (see above 1. *Thymine*).

iii. *Purine nucleoside phosphorylase* [E.C.2.4.2.1]

The *in vitro* anabolism of adenine to adenosine has been demonstrated using purine nucleoside phosphorylase from bacteria (Robertson and Hoffee, 1973) and mammals (Zimmerman *et al.*, 1971), although the mammalian enzyme has very low affinity for adenine. However, Becker (1974b) failed to detect similar activity in extracts of *Drosophila*.

The enzyme reacts in the following manner:



For general properties of the enzyme see 1. *Hypoxanthine* (above).

In vivo activity was elegantly demonstrated by Hoffmeyer and Neuhard (1971) using purine auxotrophs in *S. typhimurium*. Since this

bacterium naturally lacks adenine and AMP deaminases (see Fig. 5), two routes are available for purine auxotrophs to synthesize GMP and IMP from adenine. The first is *via* anabolic function of purine nucleoside phosphorylase; the other is through APRT and the histidine pathway *via* the formation of aminoimidazole carboxamide ribotide (an intermediate in *de novo* biosynthesis) from ATP. The last route was estimated to contribute 50% of GTP in auxotrophs and is inhibited by excess histidine. Hence, in the presence of excess histidine the auxotrophs have suboptimal growth on low concentrations of adenine. The limitation was overcome by increased availability of (d)R-1-P provided either nutritionally (thymidine or uridine) or by an additional mutation in deoxyribomutase (see Fig. 3) suggesting the existence of anabolism of adenine to adenosine by purine nucleoside phosphorylase. This was confirmed when lethality was observed upon introducing mutations of this enzyme under the same conditions.

iv. *Trans-N-deoxyribosylase* [E.C.2.4.2.6]

In *Lactobacilli*, this enzyme will convert adenine to deoxyadenosine provided that a deoxyribonucleoside is available for the reaction to proceed (see 1. *Thymine*).

v. *Adenine deaminase* [E.C.3.5.4.2]

This enzyme specifically deaminates adenine to hypoxanthine in the following manner (see Henderson and Paterson, 1973):



Successful supplementation of *S. typhimurium* purine auxotrophs by exogenous adenine in the presence of excess histidine (see above) was taken by Zimmerman and Magasanik (1964) as evidence for the existence of adenine deaminase in this bacterium. However, as described previously, Hoffmeyer and Neuhard (1971) found that an additional mutation, in purine nucleoside phosphorylase, prevented growth on adenine. Purine auxotrophs, with additional deficiencies in adenosine deaminase and SAMP synthetase, (see Fig. 5) were shown to have an absolute requirement for both adenine and hypoxanthine in the presence of histidine. Thus they concluded the absence of adenine deaminase from *S. typhimurium*. The enzyme is also absent from mammalian cells (see Murray *et al.*, 1970) and *E. coli* (Koch and Vallee, 1959) but present in other bacterial species (see Schramm and Lazorik, 1975).

Becker (1974b) reported the presence of adenine deaminase in extracts of *Drosophila*. However, Hodge and Glassman (1967a) did not observe such activity and indicated that hypoxanthine appears only after the formation of AMP, IMP and inosine.

4. Adenosine

Adenosine was shown to satisfy purine requirements in purine auxotrophs of bacteria (Zimmerman and Magasanik, 1964; Hoffmeyer and Neuhard, 1971) and *Drosophila* (Falk and Nash, 1974a; Naguib, 1976). It was also shown that ¹⁴C-adenosine fed to *Drosophila* larvae is incorporated into their nucleic acids (Johnson *et al.*, 1976).

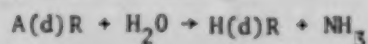
Maguire *et al.* (1972) reported that, in animals, adenosine is either phosphorylated to AMP by adenosine kinase or deaminated to

inosine by adenosine deaminase. Less often adenosine can be cleaved to adenine, by purine nucleoside phosphorylase, to be reutilized by APRT.

Green and Ishii (1972) proposed that deamination overwhelms the kinase activity with regard to exogenous adenosine and *vice versa* for internally produced adenosine.

i. Adenosine deaminase [E.C.3.5.4.4]

The enzyme has been described in different cell types. It acts in the following manner (see Henderson and Paterson, 1973).



In bacteria, the enzyme is induced by adenine, hypoxanthine and their nucleosides (Remy and Love, 1968; Jochimsen *et al.*, 1976). Deamination is quite rapid (Mans and Koch, 1960) and is the major route of adenosine and deoxyadenosine utilization (90%) in these organisms. (Hoffmeyer and Neuhard, 1971). Zimmerman and Magasanik (1964) found that growth of mutants, defective in the conversion of IMP to AMP (see Fig. 5), on adenosine or deoxyadenosine is extremely slow. These compounds are rapidly deaminated to hypoxanthine and deoxyinosine, which such mutants cannot utilize. Mutants in adenosine deaminase can be selected in such genetic background in *S. typhimurium* (Hoffmeyer and Neuhard, 1971) and *E. coli* (Jochimsen *et al.*, 1975), since they grow more rapidly on adenosine.

Adenosine deaminase activity was detected in *Drosophila* by Wagner and Mitchell (1948), Hodge and Glassman (1967 a and b) and

Becker (1974b).

Human erythrocyte adenosine deaminase has been purified by Schrader *et al.* (1976). The enzyme is genetically polymorphic (Hirschhorn *et al.*, 1973). Agarwal *et al.* (1975) reported that the enzyme is inhibited competitively by inosine.

Total absence of the enzyme was found to be associated with "severe combined immunodeficiency", a serious inherited disorder (Giblett *et al.*, 1972). However Creagen *et al.* (1973) located the genes controlling the immunodeficiency and the synthesis of the deaminase on separate chromosomes. Trotta *et al.* (1976) suggested the resolution of the paradox to be that immunodeficient individuals produce an adenosine deaminase inhibitor.

ii. Adenosine kinase [E.C.2.7.1.20]

This enzyme has been isolated from many cell types and is the major purine nucleoside kinase in animal tissues (see Murray *et al.*, 1970). It phosphorylates adenosine in the following manner:



The animal enzyme also reacts with deoxyadenosine and several analogues, but not with inosine or thioinosine (see Murray *et al.*, *op. cit.*). However, substrate specificity varies in different tissues (Snyder and Henderson, 1973). ATP, GTP and ITP all act as phosphate donors. The enzyme is not feedback inhibited (Hochstadt, 1974).

Adenosine kinase has been detected in *Drosophila* cell extracts (Becker, 1974b) and is possibly present in *Musca domestica* (Miller and Collins, 1973). However it seems to be absent from *S. typhimurium*, since purine auxotrophs lacking, in addition, purine nucleoside phosphorylase and adenosine deaminase are unable to grow on adenosine (Hoffmeyer and Neuhaed, 1971).

Green and Ishii (1972) proposed that adenosine kinase maintains the correct AMP/GMP ratio in animals. Its absence could cause an imbalance in this ratio by the accumulation of GMP due to the back flow of AMP to GMP via IMP (see Fig. 5), the rapid adenosine cycle (Balis, 1968) ($AR \rightarrow HR \rightarrow H \rightarrow IMP \rightarrow SAMP \rightarrow AMP \rightarrow AR$) and the inability of mammalian cells (lacking GMP reductase) to convert GMP to AMP. The excess GMP would be degraded, causing excretion of excess purine derivatives. Increased *de novo* biosynthesis would compensate for AMP deficiency. These latter are the symptoms of gout.

iii. *Nucleoside phosphotransferase* [E.C.2.7.1.77]

This enzyme, utilizing a low energy phosphate donor, converts adenosine to AMP (see above 2. *Thymidine*).

iv. *Purine nucleoside phosphorylase* [E.C.2.4.2.1]

General characteristics of the enzyme were discussed above (see 1. *Hypoxanthine*) and only its function with adenosine will be discussed here.

Mammalian enzyme has very low but measurable activity with adenosine and deoxyadenosine (Zimmerman *et al.*, 1970; Snyder and Henderson,

1973). Activity of this enzyme with adenosine was not detected in *Drosophila* cell extracts (Becker, 1974b) and seems to be absent from *Musca domestica* (Miller and Collins, 1973). Nevertheless, the catabolic importance of this enzyme was demonstrated by Hoffmeyer and Neuhard (1971) in *S. typhimurium* which is naturally very low in adenosine kinase (see above); purine auxotrophs lacking adenosine deaminase and purine nucleoside phosphorylase are unable to grow on adenosine as a sole purine source.

v. *Nucleoside ribohydrolase* [E.C.2.2.3]

The enzyme cleaves adenosine hydrolytically to adenine (see 4. *Uridine*).

5. Deoxyadenosine

In mammals, deoxyadenosine can be anabolized to dAMP by deoxycytidine kinase (Krenitsky *et al.*, 1976) or adenosine kinase (Snyder and Henderson, 1973) depending on tissue under investigation. Deoxyadenosine can also be deaminated to deoxyinosine by adenosine deaminase or, less likely, cleaved to adenine by purine nucleoside phosphorylase. Snyder and Henderson (1973) showed that the various animal tissues exhibit different preferences for each of these routes.

In bacteria deoxyadenosine was shown to satisfy the purine requirements of mutants lacking adenosine deaminase and adenylosuccinate (SAMP) synthetase (Hoffmeyer and Neuhard, 1971) and to be an excellent source for dR-1-P needed for thymine utilization (see 1. *Thymine*). Nevertheless, no kinase activity towards

deoxyadenosine was detected in *E. coli* (Karlstrom, 1970) or *S. typhimurium* (Hoffmeyer and Neuhard, 1971). In *Lactobacilli* deoxyadenosine is phosphorylated by deoxycytidine kinase (Durham and Ives, 1971).

In insects the only work available on this compound is by Berry and Firshein (1967) and Freeman *et al.* (1972) on *Cecropia*. Injection of ^{14}C -deoxyadenosine in pupae resulted in labelling of both guanine and adenine moieties of DNA. Deoxyadenosine is rapidly deaminated by *Cecropia* homogenates and no kinase activity was detectable.

i. *Deoxycytidine kinase* [E.C.2.7.1.74]

The enzyme from calf thymus and *Lactobacilli* converts deoxyadenosine to dAMP (see 8. *Deoxycytidine*).

ii. *Adenosine kinase* [E.C.2.7.1.20]

This enzyme converts deoxyadenosine to dAMP in animal tissues (see 4. *Adenosine*).

iii. *Nucleoside phosphotransferase* [E.C.2.7.1.77]

The enzyme catalyzes the conversion of deoxyadenosine to dAMP using low energy phosphate donors (see 2. *Thymidine*).

iv. *Adenosine deaminase* [E.C.3.5.4.4]

The enzyme deaminates deoxyadenosine to deoxyinosine (see 4. *Adenosine*).

v. *Purine nucleoside phosphorylase* [E.C.2.4.21]

This enzyme cleaves deoxyadenosine phosphorolytically to adenine and dR-1-P (see 4. *Adenosine*).

vi. *Trans-N-deoxyribosylase* [E.C.2.4.2.6]

In *Lactobacilli* deoxyadenosine, in the presence of a nucleic acid base, is converted by this enzyme to adenine (see 1. *Thymine*).

6. *Guanine*

Guanine was shown to satisfy the purine requirement of auxotrophs in bacteria (Hoffmeyer and Neuhard, 1971). Johnson *et al.* (1976) showed some incorporation of ^{14}C -guanine into nucleic acids of *Drosophila* larvae. On the other hand, no significant incorporation of guanine was detected in nucleic acids of *Drosophila* cells in culture (Becker 1974 a and b), the butterfly *Pieris brassicae* (Lafont and Pernetier, 1975) or *Musca domestica* (Miller and Collins, 1973).

In general, guanine can be converted directly to GMP by HGPRT in animal cells or by a specific GPRT in bacteria (Jochimsen *et al.*, 1975). It can also be anabolized to guanosine or deoxyguanosine by purine nucleoside phosphorylase when (d)R-1-P is available. In *Lactobacilli* guanine can be converted to deoxyguanosine by the enzyme *trans-N-deoxyribosylase*. Guanine is also deaminated to xanthine by guanine deaminase.

i. *Guanine phosphoribosyltransferase* [E.C.2.4.2.8]

Guanine can be anabolized directly to GMP by HGPRT in animal cells or by a specific GPRT in bacteria. The general properties of both enzymes are similar (see 1. *Hypoxanthine*) with the exception of substrate specificity. GPRT converts the majority of guanine (90%) to GMP and only 25-35% of hypoxanthine. HGPRT converts hypoxanthine, guanine and xanthine to their respective ribotides (Murray *et al.*, 1970). The enzyme seems to be absent from *Drosophila* cells in culture (Becker, 1974 a and b), *Musca domestica* (Miller and Collins, 1973) and the butterfly *Pieris brassicae* (Lafont and Pennetier, 1975). Furthermore, the dipterans are naturally resistant to 8-azaguanine, 6-mercaptopurine and other guanine analogues. Becker (1974 a and b) confirmed the absence of the enzyme in *Drosophila* cell extracts.

ii. *Purine nucleoside phosphorylase* [E.C.2.4.2.1]

The *in vitro* anabolic role of the enzyme was mentioned above (see 1. *Hypoxanthine*). Raivio and Seegmiller (1973) reported that after feeding ^{14}C -guanine to both normal and HGPRT deficient fibroblasts, 21-25% of total radioactivity was located in guanosine, implicating this enzyme in the synthesis of guanosine.

No anabolic activity of purine nucleoside phosphorylase was detected in extracts of *Drosophila* (Becker, 1974b). This function seems to be absent in *M. domestica* (Miller and Collins, 1973) and *P. brassicae* (Lafont and Pennetier, 1975), since guanosine but not guanine is incorporated into their nucleic acids.

iii. *Guanine deaminase* [E.C.3.5.4.3]

This enzyme deaminates guanine to xanthine; 8-azaguanine is also a substrate. It has been identified in *Drosophila* (Seecof, 1961; Morita, 1964; Hodge and Glassman, 1967 a and b; Becker, 1974b) and in mammalian tissues (see Henderson and Paterson, 1973). Josan and Krishnan (1968) found the enzyme activity in rat tissue is actively regulated by GTP, as a stimulant, and a protein inhibitor. The activity of both the enzyme and its inhibitor were shown to change with age after birth of rats (Kubar, 1969). The general presence of guanosine deaminase in animal tissues and its effective competition with HGPRT for guanine may account for the ineffectiveness of this base as a nucleic acid precursor. Raivio and Seegmiller (1973) showed that 10-26% of total radioactivity provided by ^{14}C -guanine was located in xanthine in normal fibroblast cells, and twice as much in HGPRT deficient cells. Johnson *et al.* (1976) found that 49% of dietary ^{14}C -guanine was deaminated in *Drosophila* larvae, while only 11% entered the nucleotide pool. Hodge and Glassman (1967a) reported that larval extracts of the "Canton-S" strain of *Drosophila* are unable to deaminate guanine, unless tyrosinase activity is inhibited by sodium diethyldithiocarbamate (Hodge and Glassman, 1967b). A number of other strains carrying the mutants *white*, *echinus* (*w*, *ec*), *brown* (*bw*) and *ma-l* were shown to have reduced amounts of guanosine deaminase, compared with "Pacific" wild-type (Hodge and Glassman, 1967b).

iv. Trans-N-deoxyribosylase [E.C.2.4.2.6]

In the presence of a deoxyribonucleoside this enzyme from *Lactobacilli* can convert guanine to deoxyguanosine (see 1. Thymine).

7. Guanosine

Guanosine can be anabolized to GMP by guanosine kinase or nucleoside phosphotransferase, but the main route of utilization is usually considered to involve cleavage of the glycosidic bond, by purine nucleoside phosphorylase in most cells or by hydrolases in some bacteria, to yield guanine.

i. Guanosine kinase [E.C. none]

Pierre and Le Page (1968) and Pierre *et al.* (1967) showed that guanosine is converted to GMP in crude extracts of ascites tumor cells lacking HGPRT. Zimmerman and Magasanik (1964) and Hoffmeyer and Neuhard (1971) found that combining purine auxotrophy with deficiency in GPRT or purine nucleoside phosphorylase resulted in strains able to grow on guanosine. Such results were taken as evidence for the presence of guanosine kinase. Mutants in guanosine kinase were isolated and studied in *S. typhimurium* (Hoffmeyer and Neuhard, 1971) and *E. coli* (Jochimsen *et al.*, 1975). The selection and phenotypic recognition of these mutants were done in a background of purine nucleoside phosphorylase deficiency to block this main route of guanosine utilization (see above). The mutants in *E. coli* are able to phosphorylate both guanosine and inosine. Jochimsen *et al.* (1975) also showed that the synthesis of the enzyme is not influenced by

addition of nucleosides to the medium; however, if purine auxotrophs are starved for purines, a three-fold elevation in guanosine kinase activity is observed.

Becker (1974b) did not detect guanosine kinase activity in extracts of *Drosophila* and his cell line was naturally resistant to thioguanosine. On the other hand the results of Miller and Collins (1973) on *M. domestica* and Lafont and Pennetier (1975) on *P. brassicae* indicate the presence of guanosine kinase. Both insects incorporated guanosine but not guanine into their nucleic acids. Furthermore Johnson *et al.* (1976) found that dietary guanosine is more effective than guanine in labelling nucleic acids of *Drosophila* larvae.

ii. Nucleoside phosphotransferase [E.C.2.7.1.77]

In different cell types this enzyme can phosphorylate guanosine to GMP (see 2. Thymidine).

iii. Purine nucleoside phosphorylase [E.C.2.4.2.1]

This enzyme cleaves guanosine and other purine nucleosides to their respective bases (see above 1. Hypoxanthine). Purine auxotrophs of *E. coli* lacking this enzyme grow poorly on guanosine (Jochimsen *et al.*, 1975) suggesting its importance for nucleoside utilization by bacteria. Extracts of *Drosophila* were found to cleave guanosine poorly (Becker, 1974b).

iv. *Nucleoside ribohydrolase* [E.C.3.2.2.3]

In bacteria, this enzyme cleaves guanosine hydrolytically to guanine (see 4. *Uridine*).

8. Deoxyguanosine

Deoxyguanosine was shown to satisfy purine requirements of auxotrophs in *E. coli* (Karlstrom, 1968). However, this compound does not satisfy the guanine requirements of purine auxotrophs having an additional mutation in purine nucleoside phosphorylase in *E. coli* (Karlstrom, 1970) and *S. typhimurium* (Hoffmeyer and Neuhard, 1971). This result suggests the absence of deoxyguanosine kinase activity and that the major route of utilization is *via* purine nucleoside phosphorylase in these bacteria. The conclusion is compatible with the observation that deoxyguanosine stimulates thymine incorporation into *E. coli* DNA by providing dR-1-P (Kammen, 1967). However, Durham and Ives (1971) found the deoxycytidine kinase from *Lactobacilli* is capable of phosphorylating deoxyguanosine to dGMP, as is the case with the enzyme from calf thymus (Krenitsky *et al.*, 1976). *Lactobacilli* are also capable of cleaving deoxyguanosine to guanine, in the presence of a nucleic acid base by *trans-N*-deoxyribosylase.

In *Cecropia*, no label was detected in DNA when ^{14}C -deoxyguanosine was injected into pupae (Freeman *et al.*, 1972), nor was kinase activity detected in homogenates of *Cecropia* tissue (Berry and Firshein, 1967). It should be recalled that *Cecropia* also lacks deoxycytidine kinase activity. However, Berry and Firshein (1967) reported that *Cecropia* homogenates deaminated deoxyguanosine to

deoxyxanthosine.

i. *Deoxycytidine kinase* [E.C.2.7.1.74]

The enzyme converts deoxyguanosine to dGMP in *Lactobacilli* and calf thymus (see 8. *Deoxycytidine*).

ii. *Purine nucleoside phosphorylase* [E.C.2.4.2.1]

This enzyme cleaves deoxyguanosine phosphorolytically to guanine and dR-1-P. It is the main route for utilization of deoxyguanosine in *S. typhimurium* and *E. coli* since no kinase activity is present. For general characteristics of the enzyme see 1. *Hypoxanthine*.

iii. *Trans-N-deoxyribosylase* [E.C.2.4.2.6]

The enzyme cleaves deoxyguanosine to guanine in the presence of a nucleic acid base (see 1. *Thymine*).

9. Xanthine

This base can probably be converted directly to XMP by HGPRT in animals and GPRT in bacteria. It can also be anabolized to xanthosine by animal purine nucleoside phosphorylase (see 1. *Hypoxanthine* for both reactions)

10. Xanthosine

No kinase activity with xanthosine has been reported. Thus, if xanthosine is utilized it is initially cleaved to xanthine by purine nucleoside phosphorylase (see 1. *Hypoxanthine*).

IV. BASE AND NUCLEOSIDE ANALOGUES

A. General

The usefulness of toxic analogues as tools in studying genetics and biochemistry has its origin with the isolation of resistant mutants as has been discussed above. Since analogues are often metabolized by the same enzymes and in the same manner as the natural compounds, the lesions resulting in analogue resistance are commonly quite specific. These mutants also prove to be valuable tools in studying somatic cell genetics and regulation in microorganisms as well as the genetics of resistance *per se*. They also helped in formulating different analogue combinations for cancer chemotherapy, particularly with the aim of minimizing the probability of generating resistance in neoplastic cells.

Resistance mutants may also clarify biochemical relationships and gene functions that are not recognizable by other means unless genetically controlled modifications are available; for example the evidence for the anabolic function of thymidine phosphorylase was not realized until *thy*⁻ mutants were isolated as resistant to anti-folate drugs. Another example is the several studies utilizing FU-resistant mutants, as initiated by Lacroute (1968), to investigate the regulation of the multifunctional complex harbouring the first two enzymes in pyrimidine *de novo* biosynthesis in various organisms (see for example Denis-Duphil and Kaplan, 1976; Makoff and Radford, 1976).

1. Activation of analogues

The toxicity of base and nucleoside analogues is not always due to compounds *per se*. In most cases they have to be converted to nucleotides for the expression of toxicity. As nucleotides they either inhibit enzymes of nucleotide metabolism or, as nucleoside triphosphates, become incorporated into nucleic acids, causing lethality *via* disruption of nucleic acid function.

2. Mechanisms of resistance

The literature on mechanisms of analogue resistance is voluminous and has been reviewed recently by Brockman (1974). Only central points will be mentioned here.

Resistance to analogues is acquired either physiologically, genetically, or both. The genetic form of resistance is characterized by its stability through many generations and can arise in several ways.

Since the toxic form of the base or nucleoside analogue is almost always the nucleotide form, a mutation in the enzyme responsible for converting the analogue to its nucleotide could give rise to resistance. This could occur either by decreased activity of this enzyme, alteration in its substrate specificity (so that it can react with the natural substrate but not the analogue) or total absence of the enzyme. Resistance can also arise by lack of active incorporation of the analogue nucleotide into the nucleic acid of the cell. Such a mechanism could occur either by producing an excess of the

natural substrate to compete with the analogue for the enzyme converting both to the nucleotide level, or by the failure to metabolize the analogue nucleotide to the triphosphate level. This type of resistance could be caused by any type of enzyme mutation mentioned above. Mutations in the transport system would cause the inability of the analogue to gain entrance to the cell or to the site of its activation within the cell. Increased activity of enzymes degrading the analogue or its derivatives to non-toxic forms would also give rise to resistance.

B. 5-Fluoro-2'-deoxyuridine

This deoxyuridine analogue has been widely used in cancer chemotherapy and selection of different resistant mutants in various cell types. Studies on the biological, chemical and pharmacological effects of FUDR are quite numerous and have been dealt with in several reviews, most recently by Heidelberger (1975). Hence only areas which are directly related to this work will be mentioned here.

FUDR has the same structure as deoxyuridine except for the presence of a fluorine atom at the 5 position instead of the natural hydrogen atom. The fluorine atom increases the acidity of FUDR and its derivatives and causes them to bind more strongly to enzymes than do the normal substrates and to react as cytosine in base pairing with guanine. Furthermore the small size of fluorine atom causes the fluorinated base to act as uracil rather than a thymine analogue. Thus FUTP becomes incorporated into RNA but not DNA, and FdUMP binds to thymidylate synthetase in competition with its normal substrate

dUMP (Heidelberger, 1975).

1. Activation of FdR

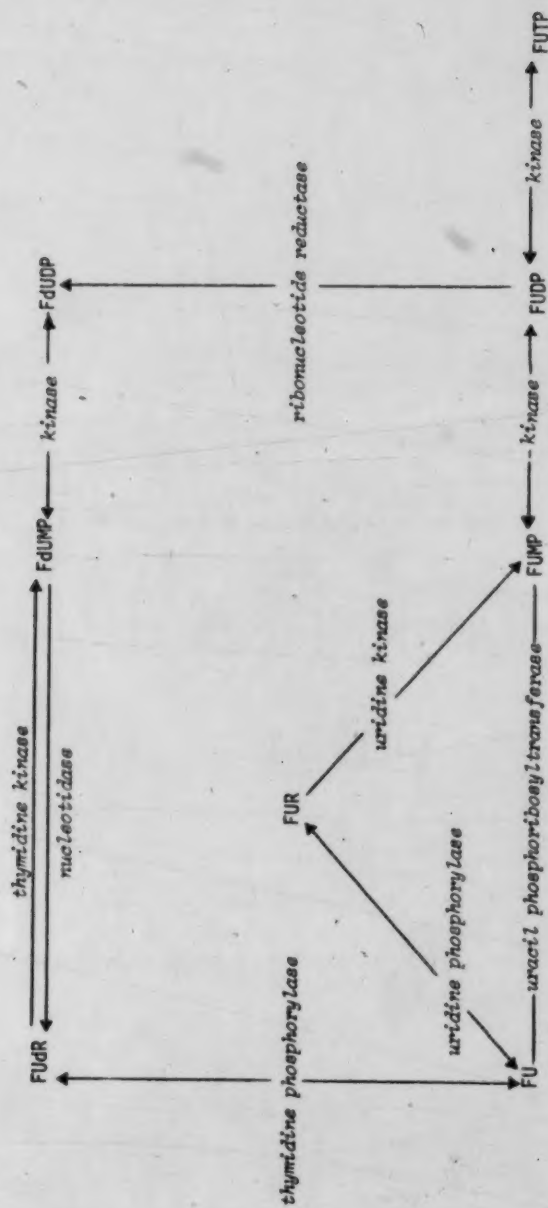
FdR has to be converted to the nucleotide level to exert its principal toxic effect. The conversion of FdR into nucleotides is carried out by the same enzymes catalyzing the conversion of deoxyuridine, as shown in Fig. 6. For details regarding the characteristics of these enzymes, see above (*S. Deoxyuridine*) and Henderson and Paterson (1973).

2. Toxicity of FdR

At low concentrations (10^{-8} - 10^{-6} M), the toxicity of FdR is mainly exerted by its nucleotide FdUMP (Salzman and Sebring, 1962). FdUMP specifically inhibits the enzyme thymidylate synthetase, thus preventing the conversion of dUMP to dTMP. In the absence of exogenously supplied thymidine, this inhibition prevents further DNA synthesis (for references see Heidelberger, 1975). The inhibition of thymidylate synthetase by FdUMP is competitive with dUMP in *Drosophila* (Carpenter, 1974) and other animal cells (Heidelberger *op. cit.*). In *Laetobacilli* FdUMP is covalently bonded with the enzyme (Santi and McHenry, 1972; Santi *et al.*, 1974; Danenberg and Heidelberger, 1976). Kinetic studies by Paul and Hagiwara (1962) showed that inhibition of DNA synthesis by low concentrations of FdR, in mammalian cells, is followed by a decrease in the synthesis of RNA and protein synthesis. Thymidine, but not uridine, overcomes such inhibition and synthesis was recovered in the same sequential order (Paul and Hagiwara, 1962; Salzman and Sebring, 1962). The efficiency of FdR as an

Figure 6. Interconversion of FUDR and its derivatives.

(Information derived from Heidelberg, 1975).



inhibitor of thymidylate synthetase is reduced, however, by its cleavage to FU by thymidine phosphorylase. This cleavage can be aborted by providing a dR-1-P donor (Yagil and Rosner, 1971).

At higher concentrations, FUDR produces other toxic effects apparently unrelated to inhibition of DNA synthesis, including a decrease in the pseudouridine content in t RNA, effects on transport and maturation of mammalian ribosomal RNA, smaller and less stable ribosomes in bacteria, fragility of bacterial cell walls and various effects on protein synthesis in different organisms (for references see Heidelberger, 1975). Such effects are mainly due to the effects of FUDR derivatives on RNA metabolism, hence, may be averted by uracil or uridine but not with thymine or thymidine (Brockman and Anderson, 1963).

In *Drosophila*, Bos *et al.* (1969) showed that dietary FUDR (5×10^{-6} M) was toxic and that lower concentrations induced morphological aberrations in the fly. These effects were prevented by the addition of thymidine. Rizki, Rizki and Douthit (1972) showed that dietary ^3H -FU is incorporated mainly into cytoplasmic RNA. In many systems, including *Drosophila* (Rizki, Douthit and Rizki, 1972) FU enhances the incorporation of BUdR into DNA as a result of thymidylate synthetase inhibition. Carpenter (1974) demonstrated the *in vitro* inhibition of the *Drosophila* enzyme by FUDR.

In other insects, Kilgore and Painter (1962) showed that ^{14}C -FU in the maternal diet is incorporated into the eggs of *M. domestica* and that viability of the eggs is very low. Hägele (1971) also found that high concentrations of dietary FUDR (10^{-4} M) caused constrictions

and partial breakage in *Chironomus* polytene chromosomes especially in areas of late replication.

3. FUdR resistant mutants

The specific inhibitory effects of FUdR render it a powerful tool in the isolation of resistant mutants with characterizeable biochemical phenotypes and it has been used for that purpose in many cell types. The most frequent resistance mutants obtained are those involved in the activation of FUdR, particularly in thymidine kinase. Other resistant mutants have been isolated, in different genetic backgrounds, in enzymes responsible for the synthesis of the 5-fluorinated uridine derivatives; that is in uracil phosphoribosyltransferase, thymidine phosphorylase, uridine kinase, and uridine phosphorylase (see above A. *The pyrimidines*). Somewhat different mechanisms account for other mutants reported. Thymidylate synthetase mutants with altered substrate specificity (Heidelberger *et al.*, 1960) or with elevated enzyme synthesis (Baskin and Rosenberg, 1975) were reported in Ehrlich ascites cells and mouse neuroblastoma respectively. Mutants in the transport of the analogue were also reported in yeast (Jund and Lacroute, 1970), bacteria (Bean and Tomasz, 1973a) and in *Aspergillus* (Palmer *et al.*, 1975). Regulatory mutants in *de novo* pyrimidine biosynthesis were also isolated as FU-resistant in different organisms (see for example Lacroute, 1968; Denis-Duphil and Kaplan, 1976; Markoff and Radford, 1976). *thy*⁻ mutants, which lack thymidylate synthetase, can be isolated as resistant to anti-folate drugs. It is to be presumed that they would also be resistant to FUdR.

V. OBJECTIVES

The aim of the present work, in part, is to examine the growth responses and toxicity levels for *Drosophila* larvae fed on all commonly occurring pyrimidine and purine bases, nucleosides and deoxynucleosides. The reason, as stated above, is the lack of a systematic study in the literature for the response of *Drosophila* and insects as a whole to these compounds.

A second, more important aspect of the work is concerned with studying the physiological effects of the deoxyuridine analogue 5-fluoro-2'-deoxyuridine (FUdR) on pyrimidine metabolism in *Drosophila*. The aim is to set up the parameters on which a FUdR-resistant mutant selection scheme could be based.

The third portion of this thesis describes attempts, which have not yet been entirely satisfying, to isolate and characterize FUdR resistant mutants.

MATERIALS AND METHODS

I. MATERIALS

A. Stocks

The different stocks used in this study are shown in Table 3.

B. Chemicals

Chemicals used in this study and their suppliers are listed below:

Sigma Chemicals Co.: Adenine; Adenosine; d-Biotin; Calcium pantothenate; Cytidine; Cytosine; 5-fluorouracil; Folic acid; Guanine; Guanosine, Deoxyadenosine; Deoxycytidine; Deoxyguanosine; Deoxyuridine; Hypoxanthine; Inosine; Nicotinic acid (Niacin); Pyridoxine HCl; Riboflavin; Ribonucleic acid (type V sodium salt); Streptomycin sulfate; Thiamine HCl (aneurin); Uracil; Uridine; Xanthine; Xanthosine.

Fisher Scientific Co.: Calcium hypochlorite; Magnesium sulfate (anhydrous); Potassium phosphate, dibasic (anhydrous); Potassium phosphate, monobasic (anhydrous); Propionic acid; Sodium bicarbonate; Sodium hydroxide; Sodium phosphate, dibasic (anhydrous); Sodium phosphate, monobasic (anhydrous).

ICN-K and K Laboratories Inc.: Ethyl methanesulfonate (EMS).

ICN-Pharmaceutical Inc.: Agar (granulated); Brewer's yeast; Sucrose.

ICN-Nutritional Biochemicals Co.: Cholesterol (scw); Lecithin (egg).

Table 3. Description of Stocks

Stock	Description*	Source
1. $Am\ Or^+$	wild type Amherst	Amherst College Amherst, Mass.
2. $Am\ Or^+/XX/Y$	$Am\ Or^+$: see above	
	XX/Y : C(1)RM, $y\ sc\ su(w^a)$ $wa\ bb/y\ sc^{4L}\ sc^{8R}$ backcrossed to $Am\ Or^+$ for 6 generations	Cal. Tech.
3. $FNC4/XX/Y$	$FNC4$: temperature sensitive lethal and female ster- ile, isolated from mut- agenized males in stock 2.	University of Alberta (U. of A.)
	XX/Y : see above	
4. $Sp/SMS, al^2$ Cy, lt^v, sp^2	Sp : strenopleural (2-22.0)	Cal. Tech.
	SMS : balancer for chromosome 2	
	Cy : Curly (2-6.1)	
	al^2 : airstaless (2-0.01)	
	lt^v : light (2-55.)	
	Sp^2 : Speck (2-.07.0)	
5. $mr\ bs^2/bw^{VI}$ ds^{33K}	mr : morula (2-106.7)	Cal. Tech.
	bs^2 : blistered (2-107.3)	
	bw^{VI} : brown-Variegated (2-104.5), also known as Plum (Pm)	
	ds^{33K} : dachsous (2-0.3)	
6. $LVM/Ly\ Sb$	LVM : In(3L) with Lethal in each arm	U. of A.
	Ly : Lyra (3-40.5)	
	Sb : Stubble (3-58.2)	
7. $C92-2$	SMS /Chromosome II recessive lethal	U. of A.

*For further information on mutants and aberrations see Lindsley and Grell (1968).

BDH Chemicals: L.B. Oxoid agar No. 3.

J.T. Backer Chemicals: Mercaptoacetic acid.

Fison Scientific Apparatus Ltd.: Casein (Fat free, Vitamin free).

Dispensaries Wholesale Ltd.: Penicillin.

Hoffman-LaRoche Ltd.: 5-fluoro-2'-deoxyuridine (Courtesy of the company).

II. METHODS

A. Preparation of the media

Media used in this study are described in Table 4. Shell vials (1 x 4 in.), caps (Kaputs, Bellco Glass Inc.) and glass beakers (if media was to be poured manually) were autoclaved for 20 min. at 121°C on wrapped cycle. They were kept in a u.v. sterile room for 24 hrs. before pouring of the media. When large batches of media were prepared, they were poured at 60°C with an automatic pipette (Brewer). The machine was flushed with 95% ethanol then with 80°C sterile distilled water prior to use. After use it was flushed by hot water first then by the ethanol. All media pouring procedures were done in u.v. sterile rooms. The vials containing the media (8 cc) were then capped and stored for several days to allow condensation to evaporate before usage.

Preparation of dead yeast - sucrose medium is fully described by Naguib (1976). With respect to defined medium, all mineral salts and vitamins were used as stock solutions and kept at 4°C. Cholesterol and

TABLE 4. Composition of Media Used

Defined Medium ⁽¹⁾			
Agar (Oxoid No. 3)	2.60 g	Biotin	0.016 mg
Casein (Vitamin Free)	5.50 g	Folic Acid	0.3 mg
Sucrose	750.00 mg	NaHCO ₃ (anhydrous)	140.00 mg
Cholesterol	30.00 mg	KH ₂ PO ₄ (anhydrous)	183.00 mg
Lecithin	400.00 mg	K ₂ HPO ₄ (anhydrous)	189.00 mg
Thiamine	0.2 mg	MgSO ₄ (anhydrous)	62.00 mg
Riboflavin	0.1 mg	Streptomycin	20.00 mg
Nicotinic Acid	1.2 mg	Penicillin ⁽²⁾	25,000 iu
Ca Pantothenate	1.6 mg	Water	To 100 ml
Pyridoxine	0.25 mg		
Dead Yeast-Sucrose Medium			
Brewers Yeast	12.5 g	When Added:	
Sucrose	10.0 g	Na ₂ HPO ₄	430.0 mg
Granulated Agar	2.0 g	NaH ₂ PO ₄	270.0 mg
Penicillin ⁽²⁾	25,000 iu		
Streptomycin	20.0 g		
Propionic Acid ⁽²⁾	1.0 ml		
Water	90 ml		
Egg-Laying Medium			
Agar	1.5 mg	Streptomycin	20 mg
Propionic Acid ⁽²⁾	1.0 ml	Penicillin ⁽²⁾	25,000 iu
Water	100 ml		
Microbial Testing Medium (YEPD)			
Agar	1.5 g	Peptone	2 g
Yeast extract	1.0 g	Dextrose	2 g
Water	100 ml		

¹Modified from Sang (1956).²Added after autoclaving

lecithin were heated in 95% ethanol to dissolve. Distilled water was added gradually replacing the evaporating alcohol. All other components and purine and pyrimidine supplements were added as dry ingredients. FUDR, however was kept as stock solution (10^{-2} M), containing 2 mg/ml streptomycin, and stored at 4°C. The medium was autoclaved at 121°C on liquid cycle. The duration of autoclaving ranged from 20 mins. for 50 mls to 45 mins. for a litre.

Microbial testing medium was supplied by courtesy of Dr. R.C. von Borstel.

B. Maintenance of axenic conditions

All experiments in this study were carried out under axenic conditions. Sterile cultures of the flies used were initially obtained by saturated calcium hypochlorite dechoriation, as described in detail by Naguib (1976). Subsequent generations were maintained on sterile yeast-sucrose medium. They were kept at 25°C in incubators which were kept exclusively for sterile cultures. To minimize infection, germfree flies were recultured in u.v. sterile rooms; handling of larvae or eggs was carried out in sterile tissue-culture hoods. Suspect cultures were checked for infection by streaking onto microbial testing medium in petri dishes.

C. Larval transfer

Approximately a week before performing a larval transfer experiment, a roll of aluminum foil paper and empty half pint milk bottles, with their mouths wrapped in aluminum foil, were autoclaved for 45

mins. at 132°C on wrapped cycle. Thereafter they were kept in a sterile u.v. room to dry out before being used. Flies (4-5 days old) were fed on fresh yeast-sucrose medium for 48 hrs. prior to egg laying. About 600 flies were then transferred to each empty sterile bottle which was then capped with a petri dish (15 x 60 mm) containing the desired medium for oviposition. Petri dishes were sealed to the bottles with masking tape and wrapped with the sterile aluminum foil. The bottles were kept upside down during oviposition. After 24 hrs., the flies were removed and the egg-laying medium was cleared of any dead flies using sterile needles. The dishes were then covered with their lids and sealed with masking tape until larvae were to be transferred. A healthy culture gave approximately 3500 larvae. Using sterile fine-pointed surgical scalpels batches of 30 larvae were transferred to shell vials containing the desired testing media. Only larvae judged to be alive at transfer were used. These either move spontaneously or in response to touch with the scalpel blade. To minimize infection, a fresh blade was used for every petri dish used. After exhausting a culture from a petri dish, it was streaked to test infection. Replicas taken from the same dish were recorded, to be discarded if the dish was shown to be infected.

All cultures were kept at 25°C, except during handling, when they were held at room temperature. Pupariation and eclosion (adult emergence) were recorded and percentages were estimated.

Two types of larval transfer experiments were performed in this study:

i. Continuous growth experiments

In this type of experiment, non-nutrient egg-laying medium (Table 4) was used. Newly hatched larvae (0-6 hrs.) were transferred to defined medium to which appropriate concentrations of purines, pyrimidines and/or analogues were incorporated.

ii. Medium shift experiments

In these experiments, nutrient medium was used for oviposition. Larvae were allowed to remain feeding on these dishes until subsequent transfer to shell vials containing a new test medium. In these experiments successive transfers of batches of 30 larvae at daily intervals were performed until this ceased to be feasible due to pupariation or death.

Because of the time length of this type of larval transfer experiment (9-10 days) and the huge number of larvae on each plate, overcrowding and mushiness of the medium were frequently observed. In such circumstances, an identical disc of medium from a fresh dish was placed on top of the medium in the crowded dish; such procedure results in the majority of larvae crawling onto the fresh medium. After approximately 10-15 mins. the fresh medium disc, with the larvae on it, was returned to the empty dish, both dishes were sealed with masking tape and returned to the incubator for further larval transfer. Repetition of this procedure was performed whenever it was deemed necessary.

D. Mutation selection

Three different protocols were used in the present study for the

isolation of FUDR-resistant mutants.

Screen 1: Selection of dominant and sex-linked resistant mutants

Mutagenic treatment and first generation cross

Am Or⁺ males (24-48 hrs. old) were fed on ethyl methanesulfonate (EMS) as described by Lewis and Bacher (1968). The concentration used was 9.6 mM, which yields 30-50% sex-linked recessive lethals (Nash, unpublished). Batches of 75 males were treated with EMS for 24 hrs. in sterile half-pint milk bottles as described by Naguib (1976). The males thereafter were transferred to empty sterile bottles to dry out for two hours. The males were then etherized and used to set up the cultures for the first generation, by mating batches of 10 males to 20 *XX/Y* virgin females (stock 3, Table 3).

The virgin females were collected using the temperature sensitive virgination technique introduced originally by Wright (1968). In an *XX/Y* stock, the paternal X-chromosome is passed exclusively to his male offspring. By the use of male parents carrying an X-linked temperature sensitive lethal, only *XX/Y* female offspring survive at the restrictive temperature (29°C) and hence will be virgins.

After four days on dead-yeast sucrose medium, the parents were transferred to defined medium containing 10^{-5} M FUDR. They were discarded after 10 days of egg-laying. Later in the course of this protocol, parents were kept only for four days on defined medium with 10^{-5} M FUDR then they were transferred to fresh defined medium within 10^{-5} M FUDR, supplemented with RNA. They were kept on the last medium

for another four days before being discarded. All cultures were maintained at 25° C and handling of flies was done under axenic conditions. Monitoring for survival among the offspring on the toxic media started 14 days after setting the cultures.

Screen 2: Selection for recessive resistant mutants on chromosome 2

Mutagenic treatment and first generation cross

Treatment of males ($Am Or^+$) was done exactly as in the previous protocol, however the concentration of 6.4 mM EMS was used at first; this was later lowered to 4.6 mM, because of the high yield (66%) of second chromosomal recessive lethals on 6.4 mM concentration. Five males were crossed to ten virgin Pm/mr females (stock 5, Table 3) in each vial. The virgin females were usually aged for approximately a week to ensure virginity. The week-old females have also the advantage of producing offspring at a more predictable time, after mating, than do younger females.

After a week on dead-yeast sucrose medium, the parents were discarded.

The second generation cross

Male offspring of the genotype $Pm/+$ were selected and mated individually to three virgin $SM5/Sp^+$ (stock 4, Table 3) on yeast-sucrose medium. Parents were discarded from successful crosses after seven days.

From this generation on, the origin and fate of each culture was recorded.

The third generation cross

SM5 males and females were separated from the *Pm* progeny and mated (3 males and 5 females) on yeast-sucrose medium. After four days they were transferred to defined medium containing thymine ($5 \times 10^{-3}M$) and FUDR. Three concentrations of FUDR were used for this scheme, $10^{-5}M$, $1.75 \times 10^{-6}M$ and $1.5 \times 10^{-6}M$. The parents were discarded after 4-5 days.

All three generations were handled under axenic condition and kept at 25°C except during handling when they were kept at room temperature.

Screen 3: Selection screen with free recombination

Mutagenic treatment and first generation cross

The treatment of males (*Am Or*⁺) was done exactly as was described in screen 2; however only one EMS concentration was used (4.3 mM). *Am Or*⁺ virgin females were used in this screen, and were aged, as described in screen 2, before mating (10 females and 5 males) on yeast-sucrose medium. The parents were discarded after a week.

The second generation cross

Individual male progeny were mated to three virgin *Am Or*⁺ females for seven days on yeast-sucrose medium before they were discarded.

Available *F*₁ daughters were included amongst the females used in this

generation. The use of these females allowed screening for any sex-linked mutants, which otherwise would have been lost.

The origin and fate of each culture was recorded from this generation on.

The third generation cross

Offspring of the second generation cross were left to mate at random and then transferred to fresh yeast-sucrose medium. Such matings should include, on average, a quarter which are between two heterozygotes carrying any given whole body mutant induced in the first generation; thus, one sixteenth of the progeny should be homozygotes for the mutant. Half of the male progeny of a female carrying a sex-linked mutant would be hemizygous for the mutant. After two days the third generation parents were transferred to defined medium with thymine ($5 \times 10^{-3}M$) and FdR ($1.5 \times 10^{-6}M$).

This selection scheme has the advantage of screening for mutants on all chromosomes and further allows free recombination, thus affording greater chance to separate desirable mutants from recessive lethals.

E. Establishment of mutant strains

Cultures which gave offspring on FdR media were first checked for infection. If they were not infected, a retest for resistance was done using sibling flies from the cultures grown on yeast medium and, if the retest proved positive, a putative mutant culture was established from the offspring surviving on FdR medium. Routine retests on defined

medium containing thymine ($5 \times 10^{-3}M$) and FUDR ($1.5 \times 10^{-6}M$) were performed every month to check retention of resistance in the mutant stocks. No definite number of parents was used in these retests (20-30 pairs of flies), and they were kept on the test medium for 3-4 days. After every monthly retest, offspring surviving the toxicity of FUDR were transferred to dead-yeast sucrose medium and used to maintain the strain after being checked for infection.

F. Characterization of the mutants

In an attempt to define the cause of resistance in the mutants, different nutritional tests were performed (see below) using continuous growth larval transfer experiments and adult oviposition. In the latter method, five males and five females per shell vial (unless stated otherwise) were permitted to feed and lay eggs for two days on the required test medium before they were removed. The number of pupae and eclosed adults was recorded and relative percentage survival was estimated. Dietary responses of the different mutant strains and their wild-type controls to the different supplements were compared.

G. Genetic mapping

In order to determine the chromosomes which carry the resistance to FUDR, males carrying the *Cy* marker on the second chromosome balancer (SM5) together with the dominant visible (and recessive lethal) markers *Ly* and *Sb* on the third chromosome were selected from a cross between stocks 4 and 6 (see Table 3). Three such males (*Cy*, *Ly* *Sb*) were mated to five virgin mutant females, on dead yeast-sucrose medium. Heterozygous male progeny carrying the three dominant markers were back-

crossed to mutant virgin females (3 males and 5 females). After three days on dead-yeast sucrose medium, they were transferred to defined medium containing thymine (5×10^{-3} M) and FUdR (1.3×10^{-6} M) for seven days.

Four genotypic classes are expected on dead-yeast sucrose medium: $Cy/2^*, 3^*/3^*$; $2^*/2^*, Ly\ Sb/3^*$; $Cy/2^*, Ly\ Sb/3^*$ and $2^*/2^*; 3^*/3^*$ (2^* and 3^* represent second and third chromosomes derived from the mutant strain).

On FUdR containing medium, *Cy* and wild type flies are expected to appear if the resistant mutant is recessive and is carried on the third chromosome. If the mutation is on the second chromosome, it is expected that *Ly Sb* and wild-type phenotypes would survive the FUdR treatment. Appearance of all four or any other combination of phenotypes would indicate dominance or interaction between different genes on the two chromosomes.

The second chromosome balancer SM5, in this mapping scheme, was later replaced by the dominant marker *Pm* because of the low viability of the *Cy*, *Ly Sb* males and to facilitate the mapping of the mutants isolated in screen 2, which all carry the *Cy* marker themselves.

H. Statistical Treatment

Statistical tests were not performed in the present study primarily because of the obvious large differences between treatments in almost all experiments. However the APPENDIX gives theoretical standard deviations for a selected series of data values at various sample

sizes used in this study.

Differences observed between or within different experiments on the same medium are almost entirely due to the freshness of the medium. To minimize artifacts due to this factor, internal controls were run with every experiment and, where a given experiment was large enough that it could not be accomplished at one time, each particular treatment was generally included each time the experiment was run. The reader should assume that a given experiment, which has been run in accordance with this rule, is associated with single control (unsupplemented medium value for survival or productivity). Direct comparisons between experiments should be made only with reference to their internal controls.

In spite of the wide use of LD_{50} (dose at which a compound kills 50% of the population exposed to it) in pharmacological studies, this metric was not used in the present investigation. Its utility would have been at its greatest in comparing the mutants with their controls. Unfortunately several mutants exhibited non-sigmoidal dose responses curves; the validity of comparison of LD values depends upon sigmoidal responses.

RESULTS AND DISCUSSION

I. RESPONSE TO DIETARY PURINES AND PYRIMIDINES

A. Results and discussion

The response of *Drosophila* to dietary supplements of nucleic acid bases and nucleosides was studied by performing continuous growth experiments. Amherst (Stock 1, Table 3) wild-type larvae were transferred to defined medium containing purine or pyrimidine bases, ribonucleosides and deoxyribonucleosides at concentrations ranging from 3.16×10^{-5} to 3.16×10^{-2} M. Tables 5 and 6 show the survival and pupariation on pyrimidine and purine related compounds, respectively. At the highest concentration used (3.16×10^{-2} M) most compounds reduced survival sharply. Hypoxanthine was the only compound which unequivocally produce no change in survival. Cytosine, thymine, thymidine, adenine, deoxyadenosine and deoxyguanosine were completely lethal. Most pyrimidine compounds were tolerated at 10^{-2} M with only cytosine and thymidine causing substantially reduced viability (23 and 30% respectively). Hypoxanthine, xanthine and probably guanine were the only purine compounds tolerated at 10^{-2} M. At lower concentrations (3.16×10^{-3} M and below) no significant deviation from controls was observed for larvae grown on pyrimidines and their derivatives except for a suggestion that the pyrimidine ribosides and bases improve survival at 10^{-3} and 3.16×10^{-4} M. Amongst the purine related compounds, inosine, adenine, adenosine and deoxyguanosine were quite toxic at 3.16×10^{-3} M. Their toxic effects were still evident at the lower concentration or 10^{-3} M.

Table 5. Percentage Survival to Eclosion and to Pupariation* Amongst Larvae Grown on Defined Medium Containing Different Pyrimidines and Pyrimidine Nucleosides

Pyrimidine Added	Molar Concentrations						
	NONE	3.16×10^{-5}	10^{-4}	3.16×10^{-4}	10^{-3}	3.16×10^{-3}	3.16×10^{-2}
Uracil	74 (77)	72 (84)	72 (85)	68 (83)	80 (86)	72 (87)	74 (81)
Uridine	70 (78)	71 (87)	65 (75)	72 (78)	81 (91)	82 (86)	83 (89)
Deoxyuridine	71 (76)	70 (76)	72 (78)	74 (79)	66 (72)	71 (74)	68 (78)
Cytosine	74 (77)	66 (81)	79 (90)	79 (86)	83 (90)	67 (78)	57 (84)
Cytidine	70 (78)	74 (78)	79 (86)	79 (85)	83 (89)	75 (87)	82 (85)
Deoxycytidine	71 (76)	74 (77)	68 (71)	72 (77)	64 (67)	69 (77)	63 (76)
Thymine	76 (80)	84 (86)	76 (76)	81 (84)	76 (80)	78 (80)	70 (73)
Thymidine	76 (80)	85 (86)	76 (78)	81 (85)	78 (81)	74 (80)	53 (63)
							0 (34)

* Survival to pupariation is shown in parentheses. Each datum is based upon a minimum of eight replicates of 30 larvae.

Table 6. Percentage Survival to Eclosion and to Pupariation* Amongst Larvae Grown of Defined Medium Containing Purines and Purine Nucleosides

Purine Added	Molar Concentrations							
	NONE	3.16×10^{-5}	10^{-4}	3.16×10^{-4}	10^{-3}	3.16×10^{-3}	10^{-2}	3.16×10^{-2}
Hypoxanthine	69 (72)	70 (75)	67 (73)	74 (78)	77 (81)	69 (77)	75 (79)	70 (83)
Inosine	58 (65)	62 (70)	66 (78)	60 (69)	37 (45)	12 (23)	30 (46)	10 (20)
Xanthine	69 (72)	60 (72)	62 (68)	57 (63)	72 (75)	54 (59)	43 (48)	21 (35)
Xanthosine	58 (65)	64 (70)	67 (72)	61 (68)	65 (71)	67 (77)	75 (83)	57 (65)
Adenine	72 (77)	69 (74)	74 (83)	67 (78)	40 (56)	11 (15)	5 (13)	0 (0)
Adenosine	74 (81)	66 (75)	62 (73)	64 (74)	41 (41)	10 (16)	4 (11)	7 (8)
Deoxyadenosine	80 (86)	77 (85)	77 (81)	77 (85)	76 (82)	59 (79)	0 (3)	0 (0)
Guanine	72 (77)	74 (84)	71 (79)	73 (82)	75 (83)	63 (69)	63 (68)	44 (52)
Guanosine	74 (81)	78 (81)	71 (75)	73 (80)	72 (76)	45 (56)	40 (66)	2 (21)
Deoxyguanosine	80 (86)	72 (79)	83 (86)	81 (88)	76 (83)	0.5 (1)	0 (0)	0 (0)

* Survival to pupariation is shown in parentheses. Each datum is based on minimum of eight replicas of 30 larvae.

It should be mentioned that some of the compounds tested, particularly guanine, guanosine and thymine, are fairly insoluble and crystallize in the medium at the higher concentrations used. This may provide protection to the larvae against their potential effects.

Pupariation data followed survivorship fairly closely in most cases, indicating that the toxic effects are manifest during the larval stage. However, in occasional instances the administration of a specific dose of a particular compound results in the lethal phase occurring after pupariation. This effect seems to be compound-specific; thus, for example, a completely lethal dose ($3.16 \times 10^{-2}M$) of thymidine gave 34% pupariation whereas the same concentration of uridine gave less pupariation (12%) but 25% of the pupae yielded adults.

Except for lower toxicity of pyrimidine as opposed to purine related compounds, no clear-cut pattern of toxicity was observed: A base may be more or less toxic than its riboside; ribosides may be more or less toxic than the corresponding deoxyribosides.

The results reported by other workers investigating growth responses of *Drosophila* to the pyrimidine and purine compounds utilized in this study are summarized in Tables 1 and 2, respectively (see INTRODUCTION Part II). Only results where the effect of a single supplement was studied are included. The apparently significant toxic effects of $9.8 \times 10^{-4}M$ uracil (Hinton *et al.*, 1951), $9.5 \times 10^{-4}M$ thymine (Hinton, 1956), $6.6 \times 10^{-4}M$ thymidine (Wolf, 1971), $7.2 \times 10^{-4}M$ xanthine and, probably, $5.8 \times 10^{-4}M$ xanthosine (Hinton, 1956) were not

repeatable under the present conditions, suggesting that they are not obligate responses of the species to these compounds. The present results showed an approximately similar mortality rate to that found by Geer (1963) using 2.4×10^{-3} M inosine. Lowered viability on 10^{-2} M thymidine (Bos *et al.*, 1969) and lethality on 2×10^{-2} M adenine (Wilson, 1942) and 2×10^{-2} thymine (Wilson, 1944) were effectively duplicated in the present results, despite the fact that these workers used a yeast-based medium.

Since the Amherst wild-type stock gives high survival even in control experiments, substantial improvements in survival could not be expected. In fact, even the maximum potential improvement of less than 50% which could have occurred in the present experiments was never found and the only consistent effect was, as reported above, the 5-15% improvement caused by certain pyrimidine related compounds at concentrations up to 10^{-3} M. The startling improvements in growth of "Oregon-K" on adenine, guanine and cytidine (Ellis, 1959) would probably indicate a strain difference, since his technique resembles the one used in this study. A less probable alternative explanation would attribute the difference to the better survival observed on the controls in the present experiments, presumably because of improvements in the basic medium introduced mainly by Bryant and Sang (1969).

Burnet and Sang (1963) studied the effects of bases and deoxyribonucleosides (at 2.88×10^{-3}) in the presence of a purine (7.2×10^{-4} M) or a pyrimidine (1.44×10^{-3} M) nucleotide. Since these experiments are in effect double supplementation studies they were not included in the literature surveyed in Tables 1 and 2. However, if the nucleotide

additives are ignored, their results differ from those in the present study (at $3.16 \times 10^{-3}M$) rather little, with the exception of adenine, which they found to allow normal levels of survival in the presence of CMP. Thus, in general, those significant effects reported by Burnet and Sang (1963) can be taken to indicate the behaviour of the unphosphorylated compounds which they used. They were not, as would have seemed possible, the outcome of interaction between the compound and a nucleotide.

Although developmental rate has been used extensively in the past as an indicator for response to dietary supplements, it has not been used in the present study for two reasons: delays in development are often correlated with toxic effects thus providing little additional information. Secondly, there is no information on whether mortality is distributed randomly over the population with respect to "biological" age. If it is not, variations in developmental rate measured by chronological age at adult emergence could be quite deceptive as estimates of 'real' biological development rate. In cases where adults eclose at times outside the normal range of controls this possibility is ruled out, and such cases do exist, although they are associated with high levels of toxicity. Thus larvae grown on near-toxic concentrations of uridine, deoxyuridine, cytidine, deoxycytidine, guanine and guanosine yield adults more than five days later than controls.

By comparison with the improvement in development rate caused by supplementation with RNA leading to the eclosion of adults about 2-3 days earlier than on unsupplemented medium, the single compounds used in this study produced relatively small improvements. Some are found at low doses of purine nucleosides and, possibly, uracil, thymine and

thymidine. Similar enhancements of development rate have been reported by other authors for most of these compounds. They have been interpreted by Sang (1957) as evidence for utilization of the effective compound by *Drosophila* and, hence, of appropriate salvage pathways. Sang (1957) and Burnet and Sang (1963) also argued the converse; thus they suggested that guanine, all pyrimidine bases and ribosides and all deoxyribonucleosides with the exception of deoxyadenosine are not utilized for "growth" by *Drosophila* larvae. Their conclusion was based upon the inability of these compounds to increase development rate, a criterion which clearly cannot be equated automatically with the lack of utilization. Confirmation of the utilization of a dietary supplement by their technique can only be obtained if the availability of the compound in question limits development rate under the experimental conditions employed. Since *Drosophila* is capable of *de novo* nucleotide biosynthesis, this criterion need not apply to all nucleotide precursors. Put simply, the effect of a certain supplement on growth rate may indicate utilization if development rate is increased, but lack of an effect cannot be taken to imply the converse. In addition, several experimental results contradict their conclusions. Some compounds which do not enhance growth under normal conditions can clearly be shown to be utilized when the conditions are altered. For example, thymidine and thymine were shown to slow development (Sang, 1957; Burnet and Sang, 1963), but when a limitation was imposed on the *de novo* dTMP biosynthesis by antimetabolites, their utilization to avert such deficiency was clearly demonstrable (Goldsmith and Harnly, 1950; Schultz, 1956; Riziki and Riziki, 1973). Moreover almost all the compounds reported not to be utilized by *Drosophila* by Sang (1957)

and Burnet and Sang (1963) with the exception of cytosine and probably guanine were proved to be utilized by *Drosophila* by a number of authors using different approaches: Enzyme assays (Wagner and Mitchell, 1948; Morita, 1964; Hodge and Glassman, 1967b; Becker, 1974 a and b; Clynes and Duke, 1975; Naguib, 1976) tracer studies in intact cells and organisms (McMaster-Kaye and Taylor, 1959; Rizki, Douthit and Rizki, 1972; Alonso, 1973; Sayles *et al.*, 1973; Becker, 1974a; Carpenter, 1974; Stroman, 1974; Johnson *et al.*, 1976) and, somewhat less directly, in studies using auxotrophs (Vyse and Nash, 1969; Vyse and Sang, 1970; Norby, 1970; Falk and Nash, 1974 a and b; Naguib, 1976; Naguib and Nash, 1976).

Somewhat surprisingly, systematic dietary tests on these compounds have not been published. The rather fragmentary results in insects, which have been studied more extensively than other animals, are discussed below.

In other dipterans Brust and Fraenkel (1955) using defined medium found that dietary adenine ($3.9 \times 10^{-4}M$), guanine ($3.4 \times 10^{-4}M$) and uracil ($4.7 \times 10^{-4}M$) did not affect pupariation significantly but caused 30, 22 and 25% reduction, respectively, in adult eclosion of the blow fly *Phormia regina*. In *Pseudosarcophaga affinis*, House (1964) reported that dietary nucleic acid bases (at approximately $3.1 \times 10^{-4}M$) in defined medium increased development time, though not significantly, compared with controls. Thymine was the most effective followed by uracil, guanine, cytosine, hypoxanthine and adenine in that order. Addition of nucleosides (at about the same concentrations) resulted in somewhat similar effects. Uridine slowed development most

followed by guanosine and cytidine; however, adenosine was shown to accelerate development, though not to the same degree as RNA. On the other hand Brooks (cited by Brust and Fraenkel, 1955) found that adenine and uracil enhanced developmental rate of house fly, *Musca domestica*, larvae.

This difference in response to dietary purines and pyrimidines among the various species of Diptera could arise from the various methods used in measuring the response to these compounds. Thus, it is important to have more than one criterion of the adequacy of dietary supplements. The differences, between or within species, may also be derived from ecological adaptations of these species. Such adaptations would seem more likely to occur among non-essential dietary requirements (nucleic acids, sugars, non-essential amino acids etc.) than among essential nutrients (vitamins, sterols, amino acids), as suggested by Burnet and Sang (1963). Thus, the difference in response between *Drosophila* (see Sang, 1957) and *Phormia* (Brust and Fraenkel, 1955) could indicate quantitative differences in the ability of the two dipterans to synthesize RNA precursors which could be attributed to the nature in their diets, for, while *Drosophila* normally feeds on diet rich in RNA (yeast) *Phormia* does not.

In other insects, Hogan (1972) reported that dietary adenine ($7.4 \times 10^{-2}M$) or guanine ($6.6 \times 10^{-2}M$) in defined medium did not show any pronounced toxicity or alteration in the development time of *Tribolium castaneum* but they prevented developmental delays caused by the addition of azaguanine. On the other hand, the pyrimidines uracil ($8.9 \times 10^{-2}M$) and thymine ($8.7 \times 10^{-2}M$) were shown to accelerate larval development.

Blaustein and Schneiderman (1960) reported that in giant silkmoths, *Samia cynthia* and *Callosamia promethea*, the injection of diapausing pupae, prior to initiation of adult development, with cytosine, cytidine, thymine, thymidine, uracil, uridine, adenine and xanthine at doses up to 5 or 10 mg/g live wt., had no visible effects on the pupae or on development of adults. A number of purines proved to be toxic: xanthosine (3.0 mg) hypoxanthine (1.3 mg), guanine (1.3 mg), adenosine (1.3 mg) and guanosine (0.38 mg) prevented development of adults, prolonging pupal life (18 days for controls) and causing death. Xanthosine caused the earliest death (28 days), followed by hypoxanthine and guanosine (37 days), then adenosine (40 days) and guanine (64 days).

B. Mechanisms of toxicity

The differences in the toxic concentrations for the compounds reported here and elsewhere in the literature suggest that, in many instances, the mechanism leading to death is compound specific. Determination of the mechanism of toxicity for each compound would be rather difficult without further careful experimentation. In spite of the large number of instances of work describing toxicity and morphological aberrations attributable to purines and pyrimidines, very few attempts have been made to elucidate the mechanisms by which such effects were generated and almost none is conclusive (Henderson, personal communication). In the present instances, the theoretical possibilities are rather diverse. Since very high concentrations have been used, one cannot rule out arbitrary interactions, unrelated to the biological

roles of the compounds themselves, or more general problems, such as osmotic shock. Nonetheless, the most likely targets would be expected to be found among the processes in which purines and pyrimidines are normally involved.

Some of these latter mechanisms might be essentially coincidental: For example a compound might be toxic if it reacts with another compound involved in more than one biosynthetic process; it might thereby reduce the concentration of the second compound and curtail activity in some other metabolic pathway to sub-vital level.

Other means for production of toxic effects might be extensions of normal regulatory processes. A metabolite could feedback inhibit *de novo* biosynthesis of an essential compound, yet not furnish the cell with its requirement for that compound. Biosynthetic pathways are also subject to genetic repression, so that toxicity could be generated *via* repression, in a manner analogous to that suggested for feedback inhibition.

When capacity for *de novo* biosynthesis is limited, hyper-induction of catabolic enzymes by high concentration of an exogenous substrate could cause serious drainage of essential metabolites. Similarly, competition for the active site on enzymes related to the uptake for essential compounds, could be critical.

One last mechanism, again regulatory in nature, has been studied extensively in relation to the effect of thymidine in particular. It has been proposed that this compound produces an imbalance in DNA

precursor pools. The experimental basis for the proposal merits further discussion.

De novo biosynthesis of deoxynucleotides, in general, proceeds via the reduction of the corresponding riboside diphosphate by the enzyme ribonucleotide reductase [E.C. 1.17.4.1]. The intimate relationship between DNA synthesis and the activity of ribonucleotide reductase is indicated by the finding that one of the enzyme's two subunits (B1) is coded for by *dna F*, one of eight genes required for DNA synthesis in *E. coli*; the other subunit (B2) is coded for by another gene, *nrd B*, close to *dna F* (see Kit, 1976).

The activity and substrate specificity of ribonucleotide reductase was shown to be determined and regulated in a complex manner by the available ATP, dATP, dGTP and dTTP. Increase in the pool of any one of these nucleotides was found to have a detrimental effect on the enzymatic reduction of nucleotides and, hence, DNA synthesis. The details of these studies are too extensive to be discussed here. They have been reviewed by Henderson and Paterson (1973), Reichard (1973) and Lowe (1975) recently. However, the pertinent information required for the present discussion can be summarized as follows: excess dTTP was shown to inhibit the reduction of both uracil and cytosine nucleotides and stimulate that of guanine nucleotides; dGTP stimulates the reduction of adenine nucleotides but inhibits the reduction of guanine and cytosine nucleotides. At low concentrations dATP stimulates the reduction of both pyrimidine nucleotides, but at high concentrations it inhibits the reduction of all four kinds of nucleotides. ATP stimulates the reduction of the two pyrimidine

nucleotides.

The effect of deoxyribonucleotides on the enzyme ribonucleotide reductase is presumed to be a major cause of toxicity exerted by high concentrations of adenine, guanine and thymine deoxyribosides: Klenow (1962), Morris *et al.* (1963); Bjursell and Reichard (1973) and Lowe (1975) have demonstrated that these compounds, after phosphorylation to the nucleoside triphosphate level, cause depletion of one or more of the nucleotide precursors needed for the continuation of DNA synthesis. These nutritional results mimic the effects reported for the corresponding deoxyribotides using *in vitro* systems, as reviewed by Reichard (1973). Furthermore the toxicity of these deoxyribosides was found to be reversed upon the addition of other deoxyribosides as precursors for the depleted nucleotides (Klenow, 1962; Morris and Fischer; Morris *et al.*, 1963; Whittle, 1966; Bjursell and Reichard, 1973; Lowe, 1975). Thus the toxicity of thymidine, deoxyadenosine and deoxyguanosine observed in the present study could be interpreted in this manner.

The toxicity by deoxyuridine and low survival caused by deoxycytidine could be explained in the same manner, if it is assumed that, as in other organisms, both are effective precursors for thymidine. In fact, the order of toxicity ($CdR < UdR < TdR$) follows the same order as synthesis ($dCMP \rightarrow dUMP \rightarrow dTMP$).

High concentrations of thymidine have been reported to have other potentially deleterious effects. Thymidine (Bresnick, 1962) and dTTP (Gerhart and Schachman, 1965) were shown to feedback inhibit the enzyme aspartate transcarbamylase [E.C. 2.1.3.2] and hence *de novo*

pyrimidine biosynthesis. Since pyrimidine requirements of *Drosophila* auxotrophs cannot be satisfied by thymidine (el Kouni and Nash, unpublished results), it can be assumed that, if such inhibition of *de novo* pyrimidine synthesis by thymidine or dTTP occurs in *Drosophila*, it would result in the toxic effect observed in the present study.

Feedback inhibition may also explain the effects of guanosine and guanine in the present study. It has been demonstrated that both these compounds, like other purines, inhibit *de novo* purine biosynthesis in many cell types (for details see Henderson, 1972). Johnson *et al.* (1976) have shown that neither guanosine nor guanine is a good precursor of adenine nucleotides in *Drosophila*. Thus inhibition of *de novo* purine biosynthesis could account for their observed toxicity. The differential toxicity of guanosine could result from the fact that it is marginally less efficient as an adenine nucleotide precursor than guanine (Johnson, personal communication). On the other hand, guanine is rather less soluble than guanosine and may simply be less available as a result. It is also noticeably less efficiently converted to nucleotides, but is rather catabolized. The same argument might apply to explain the difference in toxicity of xanthine and xanthosine, although no information is available on their utilization as purine nucleoside precursors in *Drosophila*.

Although adenosine, adenine and inosine, like other purines, inhibit *de novo* biosynthesis, their toxicity would seem to involve different mechanisms than that suggested above. Adenosine, adenine and inosine were shown to be incorporated into nucleic acids of *Drosophila* (MacMaster-Kaye and Taylor, 1959; Becker, 1974 a and b; Johnson *et al.*,

1976). Furthermore, Johnson *et al.* (1976) showed that ^{14}C -adenosine and ^{14}C -inosine are incorporated equally well into both adenine and guanine nucleotides, indicating the ability of *Drosophila* to convert adenine compounds and inosine to guanine nucleotides. Hence the depletion of purines *via* inhibition of *de novo* biosynthesis by adenine compounds or inosine cannot be assumed to be the cause of toxicity. However, blocking *de novo* purine biosynthesis would prevent effective nitrogenous excretion, since *Drosophila* is uricotelic, and might prove lethal.

Feedback inhibition of *de novo* pyrimidine biosynthesis by CTP and UTP which has been observed in other organisms, cannot be held responsible for the lethality caused by the high doses of cytidine or uridine. Both compounds were shown to satisfy the requirements of pyrimidine auxotrophs in *Drosophila* (Norby, 1970; Falk and Nash, 1974 a and b).

The mechanism for the toxicity exerted by these compounds and, indeed, by purine compounds as well, could involve restriction of PRPP biosynthesis. PRPP is required for both purine and pyrimidine *de novo* biosynthesis and, at the same time is essential in several other metabolic pathways.

Several authors have indicated that adenine, guanine, cytosine, and uracil ribonucleotides inhibit the synthesis of PRPP in many cell types (for references see Henderson, 1972). Hence adenosine and adenine, after being anabolized to nucleotides could lead to the inhibition of pyrimidine *de novo* biosynthesis and eventually to death by pyrimidine starvation. This contention could be supported by the



findings of Hershfield *et al.* (1976) and Ullman *et al.* (1976) that such effects by adenosine in mammalian cells can be relieved by addition of uridine. In the same manner it can be suggested that the toxic effects of cytidine and uridine are due to inhibition of PRPP synthesis by their nucleotides and subsequently the inhibition of purine biosynthesis.

Competition for PRPP between the different pathways could also be deleterious, particularly when exogenous bases are supplied. Von Euler *et al.* (1963) found that dietary orotate caused increase in uracil nucleotide synthesis in rat liver, the effect being reversed by the addition of adenine. The converse was reported by Schultz (1956) who found dietary orotate to reverse the growth inhibitory effects of adenine on *Drosophila* larvae. Competition for PRPP was also demonstrated by Kelley *et al.* (1970) between orotate phosphoribosyltransferase [E.C. 2.4.2.10] in pyrimidine biosynthesis and PRPP- amido-transferase [E.C. 2.4.2.14] in purine biosynthesis in skin fibroblasts. The addition of orotate reduced *de novo* purine biosynthesis, as measured by the incorporation of ^{14}C -glycine, and reduced the concentration of PRPP by 15 to 43%. On the other hand, while Hershfield *et al.* (1976) showed that high concentration of adenine reduced intracellular concentration of PRPP and pyrimidine nucleotides in human lymphoblasts, these effects were not relieved by the addition of uridine. They concluded that the toxicity of adenine was not due solely to pyrimidine starvation.

Several studies (see Henderson, 1972) have indicated that adenine reduces the intracellular concentration of tetrahydrofolate (THFA) and

thiamine coenzymes. The exact enzymatic bases of such effects have not yet been described.

The limited capacity of *Drosophila* for *de novo* nucleotide biosynthesis, and especially for purines, is demonstrated by the marked improvement in growth rate produced by the addition of RNA, adenine and adenosine to defined medium (Schultz *et al.*, 1946; Villee and Bissell, 1948; Sang, 1956 and 1957). Such limitations could be accentuated by the induction of catabolic pathways. It has been shown by several workers, most recently Albrechtsen *et al.* (1976), that in *E. coli* the catabolic enzyme purine nucleoside phosphorylase is induced by cytidine. If such a situation exists in *Drosophila* it would provide another explanation for the toxicity of cytidine.

Cytosine was shown not to be utilized by *Drosophila* to satisfy the requirement of pyrimidine auxotrophs (Norby, 1970). Utilization of thymine also seems to be limited (see below). Hence the reason for the complete lethality of these compounds is obscure when seen in the framework of normal dietary utilization.

An interesting observation, not directly related to mechanisms of toxicity, may have an interesting evolutionary consequence is found in the marked high sensitivity of *Drosophila* to purine compounds. Naguib and Nash (1976) reported that purine requiring mutants have higher requirements for purines than pyrimidine auxotrophs have for pyrimidine supplements. This suggests that purines may enter the cells less easily than pyrimidine compounds, a situation which may well have arisen as protection against the toxic effects of purines. It is by no means inconceivable that, in terms of intracellular

concentration, their effects may be even more severe than these results suggest, relative to those of pyrimidine compounds.

II. 5-FLUORO-2'-DEOXYURIDINE

A. Sensitivity to FUdR

The effect of FUdR on the development of *Drosophila* was studied using larval transfer experiments. In all experiments wild-type Amherst larvae were used. Continuous growth experiments were performed to study the effect of different concentrations of FUdR on larval development. Young larvae (0-6 hr. old) were transferred from egg-laying medium to defined medium alone, or with the addition of FUdR at concentrations ranging from 10^{-8} to 10^{-3} M. Table 7 shows the rates of pupariation and eclosion in this experiment. Pupariation and eclosion rates of larvae transferred to media containing 10^{-7} M FUdR or less were not affected significantly. At the higher concentration of 10^{-6} M FUdR, substantial reduction in pupariation is observed and occasional adults eclose. Neither adults nor pupae are observed at the FUdR concentration of 10^{-5} M or higher.

Larval mortality showed a series of FUdR concentration effects ranging from larval growth ending in death without pupariation (10^{-6} M FUdR), through prolonged larval life without growth (10^{-5} M FUdR) to immediate killing of young larvae (10^{-4} M FUdR). The demonstration that no single syndrome kills larvae fed on FUdR, but that the time and state of death are dependent on the concentration of FUdR, suggests that the effect of the analogue is cumulative over a long

Table 7. Percentage Survival to Eclosion and to Pupariation* Amongst Larvae Grown on Defined Medium Containing FUDR

Nucleosides Added (5 x 10 ⁻³ M)	Molar Concentration of FUDR					
	0	10 ⁻⁸	10 ⁻⁷	10 ⁻⁶	10 ⁻⁵	10 ⁻⁴
None	78 (84)	69 (80)	78 (87)	2 (39)	0 (0)	0 (0)
Thymidine	78 (80)	70 (78)	74 (77)	72 (72)	71 (79)	0 (0)
Uridine	76 (80)	80 (87)	72 (87)	9 (70)	0 (0)	0 (0)
Thymidine and Uridine	78 (85)	78 (85)	77 (86)	77 (85)	70 (82)	0 (22)
RNA	78 (83)	65 (68)	82 (88)	44 (55)	0 (0)	0 (0)

* Survival to pupariation is shown in parentheses below survival to Eclosion. Each datum is based upon a sample of 9 replicas of 30 larvae.

period of larval life and, perhaps, pupal life too. To test this suggestion two medium shift experiments were performed.

In the first experiment eggs were deposited on defined media containing different concentrations of FUDR. The range of FUDR concentration chosen for this experiment was that at which no eclosion was observed in the previous continuous growth experiment (10^{-5} to 10^{-3} M). Larvae were transferred at daily intervals to dead yeast-sucrose medium. Table 8 shows the pupariation and eclosion rates in this experiment. Although only living larvae were transferred, very few larvae were able to pupate or eclose after being fed on FUDR for two days; thus it appears that the majority of larvae have sustained irreparable damage within 48 hrs. of hatching and feeding on FUDR. It is probable that the damage caused by FUDR occurs even earlier, because the larvae used in this experiment were derived from a 24 hr. egg laying period. Thus, some of the larvae transferred after 48 hrs. were, in fact, a little more than one day old. The approximately 50% survival of the larvae transferred after 24 hrs. supports the suggestion; if it is assumed that embryos take the average 20 hrs. to hatch and that the egg-laying rhythm was constant during the 24 hr. collection period, then the larvae used as "one-day old" were, in fact, 4 to 28 hrs. old. Hence an average larva would seem to sustain lethal damage within 16 hours after hatching.

In the second medium shift experiment, eggs were deposited on defined medium without FUDR. Larvae were transferred at daily intervals to defined medium containing FUDR at concentrations ranging from 10^{-6} to 10^{-3} M. Pupariation and eclosion rates are shown in Table 9.

Table 8. Percentage Survival to Eclosion and Pupariation* of Larvae Transferred at Different Ages from Defined FUDR Containing Medium to Dead Yeast-Sucrose Medium

FUDR (M)	Age of Larvae at the Time of Transfer (Days)								
	0	1	2	3	4	5	6	7	8
0	84 (88)	78 (83)	78 (84)	82 (88)	84 (90)	79 (86)	79 (85)	80 (89)	83 (93)
10 ⁻⁵	77 (82)	44 (55)	8 (11)	0 (1)	0 (0)	0 (0)	0 (0)	-	-
10 ⁻⁴	76 (79)	43 (47)	0 (2)	0 (0)	0 (0)	0 (0)	0 (0)	-	-
10 ⁻³	76 (84)	43 (50)	5 (13)	1 (1)	0 (0)	0 (0)	-	-	-

* Pupariation is shown in parentheses.

Data (including 0%) are based upon a minimum of eight replicas of 30 larvae. Where insufficient larvae were available for transfer the symbol - is indicated.

Table 9. Percentage Survival to Eclosion and to Pupariation* of Larvae Transferred at Different Ages From Defined Medium to Defined Medium Containing FUDR

FUDR (M)	Age of Larvae at the Time of Transfer (Days)									
	0	1	2	3	4	5	6	7	8	9
0	77 (83)	68 (77)	77 (94)	77 (88)	80 (89)	71 (82)	80 (85)	91 (98)	84 (99)	79 (95)
10 ⁻⁶	15 (43)	12 (72)	38 (87)	40 (79)	37 (75)	43 (90)	70 (84)	75 (97)	85 (99)	82 (94)
10 ⁻⁵	0 (0)	0 (0)	0 (0)	0 (53)	2 (41)	1 (53)	17 (57)	43 (77)	39 (76)	41 (78)
10 ⁻⁴	0 (0)	0 (0)	0 (0)	0 (0)	0 (9)	0 (13)	2 (8)	3 (40)	11 (42)	8 (36)
10 ⁻³	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (3)	1 (10)	6 (17)	8 (15)

*Pupariation is shown in parentheses.

Each datum is based upon a minimum of two replicas of 30 larvae. Data for larvae older than one day at transfer is based upon at least four replicas.

At all FUDR concentrations, age of larvae at transfer seems to influence their sensitivity to the analogue. Pupariation and eclosion rates are higher for older larvae. This resistance of older larvae declines with higher concentrations of FUDR. The most important result of this experiment, however, is that the lethal concentrations of FUDR are still capable of producing their toxic effects even when the transfer is performed after irreparable damage was shown to have been sustained in the previous medium shift experiment (Table 8). High lethal doses of FUDR are still capable of exerting their effects on late third instar larvae preventing them from completing development to adults. The lethality in this particular experiment, however, seems to be mainly pupal and is dependent on the age of larvae at transfer and the concentration of FUDR. The increased resistance to a specific concentration of FUDR with the advance of age correlates well with the pattern of increase in the total amount of thymidylate synthetase activity reported in developing *Drosophila* larvae (Carpenter, 1973). Nevertheless the possibility that such resistance is due to shorter exposure to the analogue cannot be ruled out.

B. Modification of FUDR sensitivity

It is generally assumed that low concentrations of FUDR mainly cause inhibition of DNA synthesis *via* the effect upon dTMP biosynthesis. The toxic effects of higher concentrations include effects on RNA metabolism (for references, see *INTRODUCTION* Part IV B 2). However no information is available as to whether the effects on RNA may assume an overriding role in a situation where an organism undergoes a major part of its development in the presence of the analogues.

It has been reported that the effects of FUDR on DNA synthesis are relieved by thymidine and that on RNA metabolism can be alleviated by uridine (for references see Brockman and Anderson, 1963). Therefore larval transfer experiments were performed to examine, systematically, the effects of different supplements on the development of *Drosophila* larvae in the presence of various concentrations of FUDR.

1. The effects of thymidine and uridine

The effects of thymidine and uridine as potential protective agents against FUDR were studied to characterize the effects of different concentrations of FUDR on RNA and DNA metabolism. $5 \times 10^{-3}M$ thymidine or uridine was used to ensure maximum survival as demonstrated in dose response experiments (see Table 5). RNA, which according to Sang (1956), improves growth rate and hence, possibly, general health of the larvae, was also used for comparison.

Table 7 shows the results of continuous growth experiments used to test these compounds. Addition of RNA (0.4%) to the medium slightly improves eclosion and pupariation at $10^{-6}M$ FUDR. The effects of uridine were generally similar to those of RNA, although uridine containing cultures produced less flies but more pupae at $10^{-6}M$ FUDR. In contrast, thymidine allows essentially normal levels of eclosion and pupariation even at ten-fold higher concentration of FUDR ($10^{-5}M$). The addition of uridine and thymidine together did not improve adult survival compared with thymidine alone, although it did lead to occasional pupariation at $10^{-4}M$ FUDR. This seems to be a real effect, which has a more striking manifestation in medium shift-experiments

(see below).

As was shown above, transfer of larvae between different media can be expected to provide a sensitive method for investigation of temporal aspects of insect nutrition and physiology, which cannot be studied during development on a single medium. This method was used to ascertain the temporal distribution of sensitivity to FUDR (see above). It was also applied to the response to FUDR in the presence of thymidine, uridine and RNA.

In the first medium shift experiment, eggs were deposited on defined medium containing FUDR and thymidine and/or uridine. RNA was also used for comparison. Larvae were then transferred at daily intervals to yeast-sucrose medium. The concentrations of FUDR used were those at which no eclosion was observed in the continuous growth experiments in the absence of thymidine. Pupariation and eclosion rates are shown in Table 10. The presence of uridine or RNA does not change the pattern of mortality found without either additive (see Table 8). The addition of thymidine (or thymidine and uridine) results in normal eclosion throughout the experiment at 10^{-5} M FUDR as would be expected from the continuous growth experiments (see Table 7). No improvement, however, is observed with similar addition at 10^{-3} M FUDR. The addition of thymidine alone at 10^{-4} M FUDR allows a low level of eclosion even amongst larvae transferred after 7 days. More strikingly, the further addition of uridine under these conditions leads to normal survival up to the seventh day and a small proportion of larvae can still give rise to adults, after being maintained for 9 days on the FUDR medium. Pupariation, in general, is more common than eclosion, but shows

Table 10. Percentage Survival to Ecdysis and to Pupariation⁽¹⁾ of Larvae Transferred at Different Ages from Defined Medium Containing FUDR and Different Supplements to Dead Yeast-Sucrose Medium

Supplements (2)	FUDR (M)	Age of Larvae at the Time of Transfer (Days)									
		0	1	2	3	4	5	6	7	8	9
Thymidine	10 ⁻⁵	86 (89)	74 (82)	84 (89)	84 (90)	79 (89)	67 (79)	76 (82)	79 (87)	70 (82)	72 (84)
	10 ⁻⁴	83 (87)	78 (81)	16 (18)	18 (19)	17 (26)	19 (43)	8 (27)	3 (18)	0 (3)	0 (0)
	10 ⁻³	77 (80)	47 (63)	8 (22)	1 (4)	0 (0)	0 (0)	-	-	-	-
Uridine	10 ⁻⁵	82 (84)	49 (54)	12 (17)	1 (1)	0 (0)	0 (0)	0 (0)	-	-	-
	10 ⁻⁴	85 (89)	36 (44)	2 (3)	0 (0)	0 (0)	0 (0)	0 (0)	-	-	-
	10 ⁻³	83 (86)	40 (56)	5 (8)	0 (0)	0 (0)	0 (0)	-	-	-	-
Thymidine and Uridine	10 ⁻⁵	87 (91)	87 (91)	83 (91)	86 (94)	84 (90)	74 (82)	77 (82)	75 (86)	82 (92)	77 (91)
	10 ⁻⁴	86 (90)	87 (88)	70 (74)	71 (75)	77 (83)	78 (84)	81 (87)	61 (77)	31 (52)	6 (34)
	10 ⁻³	77 (80)	53 (63)	9 (18)	0 (1)	0 (0)	0 (0)	-	-	-	-
RNA	10 ⁻⁵	84 (90)	37 (40)	1 (2)	1 (3)	0 (1)	0 (0)	0 (0)	-	-	-
	10 ⁻⁴	75 (80)	38 (41)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	-	-	-
	10 ⁻³	81 (84)	50 (63)	8 (19)	0 (1)	0 (0)	0 (0)	-	-	-	-

¹Pupariation is shown in parenthesis.

²Nucleosides were added at 5×10^{-3} M each, and RNA at 4 mg/ml.

Data including (0%) are based on a minimum of eight replicas of 30 larvae. Where insufficient larvae were available for transfer the symbol - is indicated.

similar concentration dependence.

In the second medium shift experiment larvae were transferred from defined medium to defined medium containing FUdR in the presence of thymidine. Table 11 shows the eclosion and pupariation percentages in this experiment. Compared to results in the absence of thymidine (Table 9), these results show that late stages (as well as early stages) of larval development are sensitive to thymidine protection at 10^{-4} M FUdR. Hence it could be concluded that the blockage of DNA synthesis by FUdR occurs at all stages of larval life.

The inconsequential effects of thymidine on FUdR at 10^{-3} M indicate that a secondary effect of FUdR, other than on DNA synthesis, is crucial at this concentration.

Results similar to those shown above have been used as evidence that the primary cause of FUdR inhibition of bacterial or animal cell growth is due to the inhibition of the enzyme thymidylate synthetase (Cohen *et al.*, 1958; Paul and Hagiwara, 1962; Salzman and Sebring, 1962). This mode of action of the analogue has been confirmed enzymologically by several authors most recently by Santi *et al.* (1974) in bacteria and by Conrad and Ruddle (1972) in Chinese hamster cells.

The present results can be interpreted in the same manner; FUdR fed at a concentration ranging from 10^{-6} M to 10^{-5} M produces an inhibition of thymidylate synthetase sufficient to kill larvae exposed to it throughout their life. This suggestion is supported by the finding that the addition of thymidine to the diet relieves the effects of the enzyme inhibition on DNA synthesis and larvae become tolerant to FUdR

Table 11. Percentage Survival to Eclosion and to Pupariation* of Larvae Transferred at Different Ages from Defined Medium to Defined Medium Containing FUDR in the Presence of Thymidine (5 x 10⁻³M)

FUDR (M)	Age of Larvae at the Time of Transfer (Days)									
	0	1	2	3	4	5	6	7	8	9
0	78 (90)	67 (78)	70 (81)	83 (92)	80 (87)	85 (93)	79 (87)	78 (93)	86 (97)	82 (96)
10 ⁻⁶	83 (97)	70 (80)	72 (80)	69 (83)	70 (80)	78 (88)	79 (85)	81 (97)	83 (93)	76 (88)
10 ⁻⁵	60 (80)	63 (82)	57 (70)	57 (77)	63 (81)	66 (88)	71 (84)	82 (96)	78 (93)	70 (92)
10 ⁻⁴	0 (0)	0 (0)	0 (4)	0 (7)	9 (39)	13 (32)	27 (49)	68 (81)	48 (70)	31 (51)
10 ⁻³	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (6)	4 (8)	7 (15)	13 (29)

* Pupariation is shown in parentheses.

Each datum is based on a minimum of two replicas of 30 larvae. Data for larvae older than one day at transfer is based upon at least four replicas.

at both these concentrations (10^{-6} and 10^{-5} M). Moreover the medium shift experiments demonstrate that the lethal effects of FUDR are spread over a long period of larval development as would be expected if they were the result of inhibition of DNA synthesis. This conclusion is further supported by the findings of Carpenter (1974). She demonstrates that maximum inhibition of *Drosophila* thymidylate synthetase occurs at FUDR concentrations ranging from 10^{-6} to 10^{-5} M. The same result was obtained by Hartman and Heidelberger (1961) using enzymes from mouse ascites cells. This correspondence between *in vitro* inhibition experiments and the present studies is rather astonishing. It suggests that neither the cell membrane nor the alimentary tract provides an effective barrier to the passage of FUDR and that the larva neither concentrates nor detoxifies FUDR.

The lethality of higher FUDR concentrations (10^{-4} to 10^{-3} M) seems to occur through a thymidine insensitive mechanism. The finding that the addition of uridine in the presence of thymidine and 10^{-4} M FUDR very substantially improves larval tolerance to the analogue suggests that RNA metabolism is possibly involved. The effect on RNA synthesis by different fluorinated derivatives of FUDR has been demonstrated by several workers (for references see Heidelberger, 1975).

The cleavage of FUDR to FU by thymidine phosphorylase (Yagil and Rosner, 1971) is the first step in the conversion of FUDR to derivatives which affect RNA metabolism. Thus to further investigate the effects of FUDR on RNA metabolism the effects of FU on larval development was studied.

2. The effects of 5-fluorouracil

To characterize the effects of different concentrations of FU a continuous growth experiment was performed. Addition of thymidine or uridine as potential protective agents was also studied. The results are shown in Table 12. FU at 10^{-4} M causes complete lethality, which, as in the case of equimolar FUDR (Table 7), cannot be relieved by the addition of thymidine (5×10^{-3} M). This finding supports the earlier conclusion that lethality at 10^{-4} M FUDR is due to the effects of its derivatives on aspects of metabolism other than dTMP synthesis.

The suggestion that the toxicity of FU at high concentration (10^{-4} M) is related to RNA metabolism is not ostensibly supported by the negative results obtained in the presence of uridine (5×10^{-3} M). However, it was observed that the lethality pattern at 10^{-4} M FU differs according to the presence or absence of uridine. Uridine causes the larvae to grow and survive for 25 days before dying without pupariation, in contrast to the immediate death observed in the absence of uridine. This observation is analogous to that found in the presence of thymidine and uridine at 10^{-4} M FUDR, where high rates of pupariation, but not adult eclosion were observed. This is especially so considering that the effective concentration of FU in the present experiment has to have been somewhat higher than in the FUDR experiment, where some FUDR has to have been phosphorylated to produce FdUMP. Thus it is not unreasonable to suggest that lethality produced by higher concentrations of FU includes effects on RNA metabolism. The inefficiency of uridine alone in counteracting such lethality could also be explained in part by the finding of Carpenter (1974) that feeding larvae

Table 12. Percentage Survival to Eclosion and to Pupariation* Amongst Larvae Grown on Defined Medium Containing Fluorouracil

Nucleoside Added (5 x 10 ⁻³ M)	Molar Concentration of Fluorouracil in Medium				
	0	10 ⁻⁷	10 ⁻⁶	10 ⁻⁵	10 ⁻⁴
None	67 (70)	70 (74)	66 (72)	63 (69)	0 (0)
Thymidine	73 (78)	67 (73)	69 (75)	78 (84)	0 (0)
Uridine	76 (87)	75 (82)	74 (82)	63 (72)	0 (0)

* Survival to pupariation is shown in parentheses. Each datum is based on a minimum of 10 replicas of 30 larvae.

on 5.75×10^{-5} M FU for 6 hrs. resulted in 59% inhibition of thymidylate synthetase. This also suggests that while studying FU effects on RNA metabolism it may well be advisable to utilize thymidine so as to counteract possible effects on DNA metabolism. Furthermore, Bardar *et al.* (1973) and Wilkinson and Pitot (1973) demonstrated that even uridine cannot relieve the effects of short exposure to FUR (4×10^{-5} M or above) on ribosomal RNA. Thus some aspects of toxicity concerned with RNA metabolism are permanent and cannot be modified by uridine.

3. The effect of different concentrations of thymidine

The above results demonstrate that thymidine at 5×10^{-3} M is unable to relieve the effects of 10^{-4} to 10^{-3} M FUDR. One possible explanation is that thymidine at this particular concentration (5×10^{-3} M) is quantitatively not sufficient to counteract the effect of such high doses of FUDR or FU on thymidylate synthetase. Hence, increased thymidine concentrations might be required to relieve the deleterious effects of high doses of FUDR on DNA synthesis. The difficulty in testing such a suggestion is the finding that higher concentrations of thymidine are also toxic (see Table 5). However, there is a possibility that the two toxic effects might prove antagonistic, resulting in improved survival at higher doses of both nucleosides together than is found when each is administered separately. Table 13 shows the results when larvae were grown on defined medium containing FUDR at concentrations ranging from 10^{-2} M to 10^{-3} M and thymidine at concentrations from 5×10^{-5} M to 5×10^{-2} M. Low concentration of thymidine (5×10^{-5} M) significantly improves eclosion at low concentration of FUDR (10^{-6} M). Eclosion is improved even more by increasing the thymidine concentration until it

Table 13. Effect of Different Concentrations of Thymidine on Percentage Survival to Eclosion and to Pupariation* Among Larvae Grown on Defined Medium Containing FUDR

Molar Concentration of Thymidine	Molar Concentration of FUDR					
	0	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
None	73 (87)	71 (87)	2 (63)	0 (0)	0 (0)	0 (0)
5×10^{-5}	78 (85)	78 (84)	43 (83)	0 (0)	0 (0)	0 (0)
5×10^{-4}	82 (87)	78 (84)	74 (85)	57 (71)	0 (1)	0 (0)
5×10^{-3}	77 (88)	77 (81)	73 (80)	71 (83)	0 (0)	0 (0)
10^{-2}	68 (84)	66 (86)	71 (89)	56 (80)	0 (0)	0 (0)
2.5×10^{-2}	2 (55)	2 (54)	2 (56)	2 (48)	0 (0)	0 (0)
5×10^{-2}	0 (4)	0 (10)	0 (7)	0 (4)	0 (0)	0 (0)

* Survival to pupariation is shown in parentheses below survival to eclosion. Each datum is based upon a minimum of 8 replicates of 30 larvae.

reaches a maximum (at 5×10^{-4} M thymidine). A similar pattern is observed at 10^{-5} M FUDR, but eclosion requires a ten-fold greater thymidine concentration (5×10^{-4} M), and maximum eclosion is reached at 5×10^{-3} M thymidine. Pupariation follows the same pattern as eclosion. However these results are negative with respect to the question at hand; very high concentrations of thymidine do not relieve the toxicity of high doses of FUDR (10^{-4} , 10^{-3} M). Indeed, survival at 10^{-2} M thymidine and 10^{-5} M FUDR is marginally less than when either compound is used at similar concentration, but with the concentration of the other somewhat reduced.

Similar results were obtained by Morris and Fischer (1963) in murine mast cell neoplasm. Thymidine below 10^{-5} M, relieved the inhibitory effects, caused by 10^{-7} M FUDR, on cell reproduction. Higher concentrations of thymidine, however, inhibited cell division even in the absence of FUDR.

Some of the experiments just described were repeated in the presence of RNA (0.4 mg/ml). The results are shown in Table 14. The substantial improvements in pupariation in the presence of 10^{-4} M FUDR and 5×10^{-4} thymidine mimic the results found with 5×10^{-3} M thymidine and uridine (see Table 7). RNA is not effective in generating pupariation in the presence of 10^{-4} M FUDR and 5×10^{-3} thymidine, although in the presence of thymidine (5×10^{-3} M and 5×10^{-2} M), larval life is significantly extended by the addition of RNA at 10^{-4} M FUDR. Both observations tend to reinforce the notion that, at higher FUDR concentrations, death is caused by intervention of FUDR in RNA metabolism.

Table 14. Effects of Different Concentrations of Thymidine on Percentage Survival to Eclosion and to Pupariation Amongst Larvae Grown on Defined Medium Containing FUDR and RNA

Molar Concentration of Thymidine	Molar Concentration of FUDR in Medium				
	0	10^{-7}	10^{-6}	10^{-5}	10^{-4}
None	69 (82)	49 (66)	29 (64)	0 (0)	0 (0)
5×10^{-5}	70 (76)	55 (81)	44 (60)	0 (0)	0 (0)
5×10^{-4}	78 (92)	51 (81)	57 (70)	49 (73)	0 (22)
5×10^{-3}	61 (91)	44 (76)	40 (68)	45 (65)	0 (0)
5×10^{-2}	0 (27)	0 (28)	0 (41)	0 (22)	0 (0)

* Survival to pupariation is shown in parentheses. Each datum is based upon a minimum of six replicas of 30 larvae.

RNA does relieve the toxic effect of 5×10^{-2} M thymidine to the extent of increasing pupariation, but this effect does not appear to depend upon the presence of FdR, other than as an overriding toxic agent.

4. The effects of deoxycytidine

The toxicities of high concentrations of FdR and thymidine, as shown above, are not mutually exclusive; however, the possibility still exists for examining the interaction between the two compounds at high concentrations if the toxicity of high concentrations of thymidine could be neutralized.

The increasing level of dTTP resulting from high thymidine concentrations (Bjursell and Reichard, 1973) causes inhibitory effects on thymidine kinase, dCMP deaminase (in animals) or dCTP deaminase (in bacteria) and ribonucleotide reductase (see Cleaver, 1967 and O'Donovan and Neuhard, 1970). The inhibition of the reductase is apparently of crucial importance, since it inhibits the further synthesis of dCTP. Deficiency in dCTP results in the inability of the cell to maintain DNA synthesis (Morris *et al.*, 1963; Bjursell and Reichard, 1973), probably causing permanent damage to chromosomes (Yang *et al.*, 1966) and reducing cell survival (Kim *et al.*, 1965) even when thymidine is removed. High levels of dTTP also inhibit aspartate carbamyltransferase, in bacteria, and, consequently, *de novo* pyrimidine biosynthesis (Gerhart and Schachman, 1965).

Morris and Fischer (1963), Morris *et al.* (1963), Whittle (1966) and Bjursell and Reichard (1973) were able to reverse the toxicity

of high concentration of thymidine in mammalian cells by the addition of deoxycytidine. Deoxycytidine can be utilized by the kinases to produce dCTP (see Fig. 1), thus by-passing the dCTP deficiency.

If it were possible to relieve the thymidine toxicity in *Drosophila* by the addition of deoxycytidine, the still open question of whether the effects of FUdR at high concentrations can be reversed by thymidine could be examined. To investigate this proposition larvae were transferred to defined medium containing FUdR (10^{-7} to 10^{-4} M) and a lethal dose of thymidine (2.5×10^{-2} M) in the presence or absence of deoxycytidine (5×10^{-4} M). The results are shown in Table 15. Deoxycytidine fails to ameliorate the effect of high doses of thymidine. Thus these results provide no reason to modify the original hypothesis that the toxicity of high doses of FUdR involves effects on RNA synthesis. On the other hand they do not add any additional support for it.

Unexpectedly, however, deoxycytidine markedly improves pupariation and eclosion in the presence of 10^{-6} M FUdR. To further investigate this interesting result, characterization of the effect of deoxycytidine on FUdR toxicity was carried out. The effects of different concentrations of deoxycytidine in the presence or absence of FUdR are shown in Table 16. Deoxycytidine at 5×10^{-5} M does not have any significant effects on survivorship or pupariation. However at 5×10^{-4} M, deoxycytidine markedly improves pupariation and eclosion at 10^{-6} M FUdR, confirming the original results. Additional increase in deoxycytidine concentration (5×10^{-3} M) proves to be even more potent, resulting in larvae surviving 10^{-5} M FUdR. This improvement, however, is less than that reported in the presence of equimolar concentrations of thymidine

Table 15. Effect of Deoxycytidine ($5 \times 10^{-4}M$) on Percentage Survival to Eclosion and to Pupariation* Among Larvae Grown on Defined Medium Containing FUDR With or Without Lethal Concentration of Thymidine ($2.5 \times 10^{-2}M$)

Nucleoside Added	Molar Concentration of FUDR			
	0	10^{-7}	10^{-6}	10^{-5}
None	69 (80)	63 (77)	0 (47)	0 (0)
Thymidine	2 (51)	2 (48)	2 (56)	2 (48)
Deoxycytidine	73 (80)	64 (78)	33 (45)	0 (0)
Thymidine and Deoxycytidine	4 (59)	6 (66)	4 (69)	2 (62)

* Survival to pupariation is shown in parentheses below survival to eclosion. Each datum is based upon a minimum of 6 replicas of 30 larvae.

Table 16. Effect of Different Concentrations of Deoxycytidine on Percentage Survival to Eclosion and to Pupariation* Among Larvae Grown on Defined Medium Containing FdR

Molar Concentration of Deoxycytidine	Molar Concentration of FdR				
	0	10^{-8}	10^{-7}	10^{-6}	10^{-5}
None	61 (75)	53 (71)	61 (78)	2 (35)	0 (0)
5×10^{-5}	63 (71)	57 (75)	57 (78)	1 (37)	0 (0)
5×10^{-4}	64 (72)	61 (73)	61 (73)	37 (60)	0 (0)
5×10^{-3}	54 (76)	56 (75)	51 (74)	48 (74)	23 (81)
					0 (0)

* Survival to pupariation is shown in parentheses below survival to eclosion. Each datum is based upon a minimum of 6 replicas of 30 larvae.

(see Table 13).

In general the utilization of deoxycytidine as a thymidine precursor is considered to occur *via* deamination of either deoxycytidine *per se* or of its phosphorylated derivatives (dCMP in higher organisms and dCTP in bacteria) to produce deoxyuridine, or the appropriate nucleotide, which is then converted to dTMP by thymidylate synthetase, the enzyme inhibited by FUdR (see Fig. 1). The question arises therefore as to why deoxycytidine is at all effective as an antidote.

At least three classes of explanation of the effect are possible: First, since thymidylate synthetase of *Drosophila* is reported to be inhibited competitively by FdUMP (Carpenter, 1974), deoxycytidine may provide an increased pool of dUMP allowing successful competition for thymidylate synthetase; secondly, deoxycytidine may indirectly alleviate the inhibition of thymidylate synthetase by detoxification of FUdR or excluding it from the cell; the third possibility is that deoxycytidine itself is converted to thymine nucleotides by some alternate pathway not involving thymidylate synthetase.

5. The effects of deoxyuridine

If deoxycytidine counteracts the toxicity of FUdR by providing excess dUMP then it might be predicted that the addition of deoxyuridine under the same conditions would itself prove to be an antidote. To test this possibility, larvae were transferred to defined media containing FUdR (10^{-7} to 10^{-4} M) and deoxyuridine (5×10^{-3} M). The results are shown in Table 17. Deoxyuridine produces a markedly lesser effect than deoxycytidine at 10^{-6} M FUdR and no effect at all

Table 17. Percentage Survival to Eclosion and Pupariation* Among Larvae Grown on Defined Medium Containing FUDR and Deoxyuridine or Deoxycytidine

Nucleoside Added , (S x 10 ⁻³ M)	Molar Concentration of FUDR			
	0	10 ⁻⁷	10 ⁻⁶	10 ⁻⁵
Deoxyuridine	85 (88)	78 (85)	60 (83)	0 (0)
Deoxycytidine	71 (83)	71 (85)	67 (82)	41 (86)
None	79 (84)	77 (88)	3 (57)	0 (0)

*Survival to pupariation is shown in parentheses below survival to eclosion. Each datum is based upon a minimum of 13 replicas of 30 larvae.

at 10^{-5} M FUdR. The finding that the efficiency with which the three pyrimidine deoxyribosides counteract FUdR toxicity (thymidine > deoxycytidine > deoxyuridine) follows a different order from the usually accepted synthetic pathway connecting their nucleotides (dCMP $\rightarrow$ dUMP $\rightarrow$ dTMP) is curious. It can be argued that the pattern and efficiency of utilization of deoxyuridine as a precursor for dUMP has not yet been tested in *Drosophila*; however it has been demonstrated that its analogue, FUdR, is effectively converted at a much lower concentration (5000-fold less) as judged by its effect on thymidylate synthetase (see Carpenter, 1974) and upon survivorship (see Table 7); the same enzyme, thymidine kinase, is presumed to mediate both dUMP and FUdUMP production.

It is possible that deoxycytidine may detoxify FUdR by causing its cleavage to FU. It has been demonstrated in bacteria that deoxyribonucleosides effectively induce thymidine phosphorylase, the enzyme responsible for the cleavage of FUdR to FU (see INTRODUCTION, Part III A 2 iii). In this case one would predict that deoxyuridine should exert similar detoxifying effects. The results in Table 17 do not seem to support this contention. However, it could be argued that since no nucleoside phosphorylase is known for deoxycytidine directly, it would remain effective longer than deoxyuridine as a phosphorylase inducer, and, at the same time, serve less effectively as a source of dR-1-P. dR-1-P is also, of course, a product of cleavage of FUdR by thymidine phosphorylase, a reaction which would therefore be expected to be reversed or inhibited by the accumulation of dR-1-P.

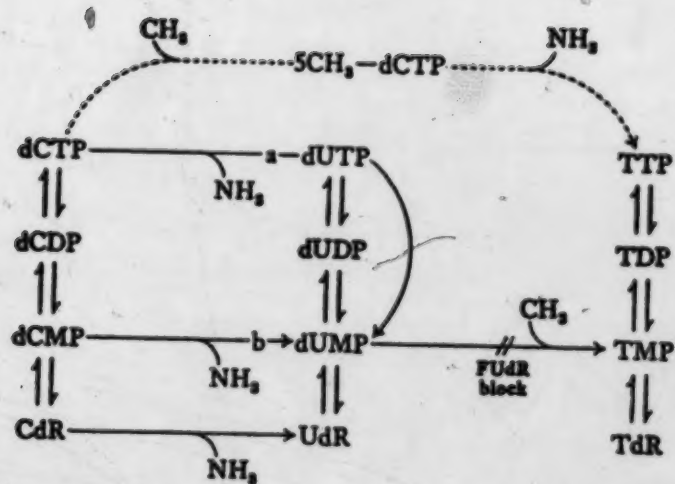
There is really no estimate of the relative degree to which the antagonistic effects of phosphorylase induction and phosphorylase activity might alter the rate of detoxification of FUDR in this system. Furthermore, since deoxycytidine is probably deaminated to produce deoxyuridine, the difference between the effects of the two compounds is presumably only a matter of degree. This difference may be reflected in the relatively greater capacity of deoxycytidine to overcome FUDR toxicity. However, Yagil and Rosner (1971) demonstrated that deoxycytidine and deoxyuridine, among other nucleosides, substantially inhibit the *in vivo* cleavage of ^3H -6-FUDR to ^3H -6-FU in bacteria, (around 50% of the counts remained in FUDR as opposed to 2.4% in controls). This inhibition of FUDR cleavage was further demonstrated in mutants lacking thymidine kinase. Although FUDR in these mutants is not converted directly to FdUMP, it still exerts toxic effects *via* its cleavage product, FU. This toxicity was reduced equally well by the addition of either deoxycytidine or deoxyuridine. Therefore both compounds are equally effective in enhancing the stability and, in their thymidine kinase mutants, reducing toxicity of FUDR. If a similar situation applies in the fruit-fly, the primary capacity of either compound to relieve the effect of FUDR is clearly not a function of detoxification.

A third possibility is that deoxycytidine is converted to dTMP without the involvement of thymidylate synthetase (see Fig. 7). A pathway for such a conversion has been suggested to exist in *E. coli* by Forster and Halldorf (cited by O'Donovan and Neuhard, 1970). They claimed that dCTP can be converted to dTTP by sequential action of a

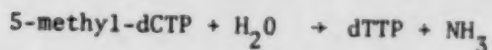
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Figure 7. Interconversion of the pyrimidine deoxynucleosides. Established reactions are shown with solid lines, postulated reactions with broken lines.

(a) In bacteria. (b) In higher organisms. The postulated methylation-deamination reaction could occur at any level of phosphorylation.



methylating enzyme and a deaminase as follows:



The methylating enzyme is reported to be separable from thymidylate synthetase on DEAE cellulose. It was not reported, however, whether the 5-methyl-dCTP deaminase is the same as dCTP deaminase. Moreover, the *thy*⁻ phenotype was described by Forster and Halldorf as a double mutant lacking both dCTP methylation enzyme and thymidylate synthetase activities. Two types of revertants were also described, one containing activity for thymidylate synthetase but none for dCTP methylation and the second showing the reverse combination.

Although this report provides a convenient explanation for the present results with deoxycytidine, it should be remembered that no definitive evidence for the dCTP methylation enzyme has been published except in thesis form.

It would be difficult to test this hypothesis by nutritional means in *Drosophila*. Feeding flies on 5-methyl-deoxycytidine in the presence of FUDR could provide evidence for its deamination to thymidine, a reaction which appears to be effective in counteracting FUDR toxicity in bacteria (Cohen and Barner, 1957) and amethopterin inhibition in mammalian cell culture (Hakala and Taylor, 1959), but will not prove that *Drosophila* is capable of methylating deoxycytidine or its phosphorylated derivatives.

The utilization of antifolate drugs would theoretically inhibit the methylation of cytosine compounds and result in death, which might be avoided by the addition of thymidine or 5-methyl-deoxycytidine, but not by deoxyuridine or deoxycytidine. The difficulty in testing this possibility would probably arise from the involvement of folate in other metabolic pathways and the obligate requirement of *Drosophila* for folate (Sang, 1956). Khan and Alderson (1968) demonstrated that folate deficiency cannot be corrected if larvae are fed on folate-free defined medium with aminopterin for more than 48 hrs., even if the larvae are subsequently transferred to defined medium containing folate and RNA.

Nevertheless manipulation of medium shift experiments under these conditions, but with provision of a utilizable purine source (suggested by the results of Morris and Fischer, 1963) could prove very valuable in exploring the possibility of methylation of deoxycytidine or its phosphorylated derivatives by *Drosophila*. Clearly the most satisfactory test would involve the demonstration of the methylation reaction in conditions under which thymidylate synthetase activity was demonstrably absent and/or the amination of thymine, thymidine and thymidylate were ruled out. Such a demonstration would, presumably, involve an isotopically labelled methyl group on the methyl donor.

6. The effect of thymine

The effectiveness of thymine in modifying FUDR toxicity was investigated in continuous growth experiments. Thymine has the potential to act as a dTTP source given appropriate biological conditions (see

INTRODUCTION Part III A 1). Larvae were transferred to defined medium containing FUDR at concentrations ranging from 10^{-7} to 10^{-4} M. The effect of different concentrations of thymine is shown in Table 18. Only high concentration of thymine (10^{-2} M) shows a significant effect on the toxicity of FUDR at 10^{-6} M, since 43% of larvae eclosed compared to none in the absence of thymine. The effect of 10^{-2} M thymine is also demonstrated at 10^{-5} M FUDR, for although no adults eclose, significant pupariation is observed. Similar results were reported by Goldsmith and Harnly (1950); only 8.3×10^{-3} M thymine or above were able to modify the toxic effect of aminopterin on adult *Drosophila*. These results demonstrate the ability of *Drosophila* to utilize thymine to overcome the inhibition of DNA synthesis exerted by FUDR. However thymine is obviously less efficient than thymidine in this respect (see Table 13). Similar conclusions were derived from comparative studies on the incorporation of thymine and thymidine into DNA of plants (Zilberstein *et al.*, 1973b), Novikoff hepatoma cells (Goodman, 1974), and silk moth pupae (Selman and Kafatos, 1974).

7. The effect of deoxyadenosine

The inefficiency of thymine utilization in *Drosophila* could be due to the limited availability of dR-1-P, required for reverse phosphorylation of thymine. This limitation in dR-1-P was shown to be overcome in bacteria (Kammen, 1967; Munch-Petersen, 1967; Budman and Pardee, 1967; Yagil and Rosner, 1970; Dale and Greenberg, 1972) and Novikoff hepatoma cells (Goodman, 1974) by the addition of a dR-1-P donor. The most efficient donors were the purine deoxyribonucleosides (Kammen, 1967).

Table 18. Effects of Different Concentrations of Thymine on Percentage Survival to Eclosion and to Pupariation* Among Larvae Grown on Defined Medium Containing FUDR

Molar Concentration of Thymine	Molar Concentration of FUDR			
	0	10^{-7}	10^{-6}	10^{-5}
None	76 (84)	73 (89)	0 (43)	0 (0)
5×10^{-5}	75 (80)	73 (75)	0.8 (53)	0 (0)
5×10^{-4}	77 (84)	76 (89)	0.4 (35)	0 (0)
5×10^{-3}	82 (84)	81 (87)	0.4 (55)	0 (0)
10^{-2}	81 (85)	77 (80)	43 (79)	0 (22)

* Survival to pupariation is shown in parentheses below survival to eclosion. Each datum is based upon a minimum of 7 replicas of 30 larvae.

If this were the situation in *Drosophila* it might be possible to enhance the effect of thymine on FUdR toxicity by the addition of a dR-1-P source. To test this suggestion larvae were transferred to defined medium containing thymine (10^{-2} M) and FUdR (10^{-6} to 10^{-4} M). The effect of deoxyadenosine was studied at a concentration of 5×10^{-4} M to avoid its own toxicity exerted at higher concentrations (see Table 6). The results are shown in Table 19. The presence of deoxyadenosine significantly enhances the effectiveness of thymine in counteracting the toxicity of 10^{-5} M FUdR. However, even with this improvement, the effect of thymine is still less than that observed when thymidine was used. At 10^{-6} M FUdR, however, deoxyadenosine reduces survivorship and pupariation. Although this toxicity of deoxyadenosine at 10^{-6} M FUdR is less pronounced in the absence of thymine, nevertheless it is accompanied by very much earlier death of the larvae than observed in controls. This interaction between deoxyadenosine and FUdR could be due to the effect of the former on preventing the cleavage of FUdR to FU, thus enhancing its toxicity. Similar results were reported by Yagil and Rosner (1971) in bacteria. The addition of deoxyadenosine prevented *in vivo* cleavage of ^3H -6-FUdR to ^3H -6-FU. It also prevented the death caused by FU, resulting from the cleavage of FUdR, in mutants deficient in thymidine kinase.

The above results probably indicate that, in *Drosophila*, as in other organisms, the inefficiency of thymine is due to the limited availability of dR-1-P and suggest the adequacy of deoxyadenosine as a dR-1-P donor under the present experimental conditions. This suggestion can be reinforced by the observation that deoxyadenosine

Table 19. Effect of Deoxyadenosine (5×10^{-4} M) on Percentage Survival to Eclosion and to Pupariation* Among Larvae Grown on Defined Medium Containing FudR With or Without Thymine (10^{-2} M)

Nucleoside Added	Molar Concentration of FudR		
	NONE	10^{-6}	10^{-4}
None	66 (73)	1 (49)	0 (0)
Thymine	61 (65)	51 (65)	0 (0)
Deoxyadenosine	76 (84)	0 (37)	0 (0)
Thymine and Deoxyadenosine	68 (74)	40 (59)	0 (0)

* Survival to pupariation is shown in parentheses below survival to eclosion. Each datum is based on a minimum of 17 replicates of 30 larvae.

probably enhances FUDR toxicity.

III. ISOLATION OF MUTANTS

The mutation selection screens used in this study are based on the assumption that all progeny of mutagenized flies will die when exposed to lethal doses of FUDR with the exception of those which have acquired resistance to the analogue. Such individuals could either be defective in enzymes involved in the metabolism of FUDR or are mutants which cause changes in substrate specificity or rate of activity of thymidylate synthetase, thus conferring resistance to FUDR. Mutants deficient in thymidylate synthetase are assumed to be lethals unless a thymidylate source is provided during selection. It has been observed, in bacteria, that *thy*⁻ mutants are more efficient in the utilization of thymine than *thy*⁺ strains even if the latter were grown in the presence of dR-1-P source. The efficiency is suggested to be due to the increased intracellular concentration of dR-1-P found in these mutant strains as a result of catabolism of accumulated deoxynucleotide precursors of dTTP, whose synthesis has been blocked by the mutation (see Beacham and Pritchard, 1971; Uerkvitz *et al.*, 1973). If such a situation exists in *Drosophila* it would facilitate the distinction between *thy*⁻ and *thy*⁺ strains grown on thymine in the presence of FUDR.

Other types of mutants can also be obtained; for example mutants in the enzyme(s) or system(s) responsible for the uptake of FUDR by the cell could also produce resistance to the analogue.

Three basic selection screens were adopted in the present study. The first was an attempt to isolate dominant or sex-linked resistance mutants (screen 1, Table 20). The rationale is that, since XX/Y females were used in this screen and the progeny are tested for resistance in the first generation, surviving male progeny could carry either dominant or recessive X-linked resistance mutants. Both male and female survivors could also carry dominant resistance mutants on the autosomes.

Although a total of 17,200 males were mutagenized, no resistant offspring were obtained (see Table 20). This failure could have several causes; the FUDR concentrations used might have been too high or some other element in the conditions under which the selection were carried out may have been inappropriate. Alternatively, it might be that neither dominant nor sex-linked recessive FUDR-resistant mutants can exist. In retrospect, the first possibility seems to be the most appropriate, since mutants were isolated when FUDR concentration was lowered (see Table 20).

Upon the failure of screen 1 to produce resistant mutants, it was decided to broaden the spectrum of the selection screen to include also the search for autosomal recessive resistance mutants. It was also decided to replace RNA in the test medium with thymine, to provide the possibility for selecting thymidylate synthetase deficient mutants as has been done successfully in bacteria using anti-folate drugs (see O'Donovan and Neuhaard, 1970).

Isolation of recessive mutants requires the generation of homozygotes, so that the recessive phenotype can be observed. In

Table 20. Summary of the Results Obtained in Different Mutation Screens

Mutation Screen	EMS [mM]	FUDR [M]	Media Supplements (1) RNA Thymine	Number Tested (2)	No. of Mutants Isolated
1	9.6	10^{-5}	-	5200	0
	9.6	5×10^{-6}	-	5600	0
	9.6	10^{-5}	+	1600	0
	9.6	5×10^{-6}	+	4800	0
2	6.4	10^{-5}	-	87	0
	6.4	7.5×10^{-6}	-	583	0
	6.4	5×10^{-6}	-	797	0
	4.3	7.5×10^{-6}	-	460	0
	4.3	1.5×10^{-6}	-	1581	9
3	4.3	1.5×10^{-6}	-	1862	3

¹RNA added at 4 mg/ml and thymine at $5 \times 10^{-3}M$.

²In screen 1 it represents number of treated males; in screen 2 and 3 it represents the number of lines tested.

Drosophila, "balancer" chromosomes are generally used to achieve homozygosity. Practically, this method can only be applied to one autosome at a time; Naguib (1976), in her search for nucleoside auxotrophs applied the balancer method to both the large autosomes, the second and the third, simultaneously and encountered numerous problems. However when she focused on the second chromosome alone, the method was successful (see Screen 2 and also Naguib, 1976).

This technique has some shortcomings. It limits the search for FUDR recessive resistant mutants mainly to the second chromosome and discards recessive mutants, when combined with recessive lethals on the second chromosome. However numerous available strains, made as by-products of Naguib's experiments, were convenient as material on which to test potential conditions for isolating recessive FUDR resistant mutants.

The early results from Screen 2, however, were not positive. Consequently, FUDR concentration was reduced (see Table 20). EMS concentration was also lowered from 9.6 mM to 6.4 mM to reduce the 67% homozygous recessive lethals observed by Naguib (1976). This concentration was further lowered to 4.3 mM when it was realized that this concentration still produces 66% recessive lethals. The last concentration lowered the recessive lethality to 50%.

Under the new conditions nine apparent mutants were isolated in screen 2. All were balanced lethal strains carrying the SM5 chromosome and a presumably induced second chromosome recessive lethal. These results suggest that recessivity was not necessarily the problem causing

the failure of screen 1 to yield resistant mutants, but more probably the high FUDR concentrations used were the cause.

It is possible to test all hypotheses by the use of screen 3. This scheme has a number of advantages over screen 2. Since only FUDR resistant mutants can survive in the presence of FUDR, then if the mutants are recessive, only homozygotes will survive; thus the use of FUDR can replace balancer chromosomes in generating homozygous recessive resistant mutants. In addition, the free recombination in screen 3 has the advantage of allowing the separation of recessive lethals from resistance mutants. Other advantages of screen 3 are that it screens for mutants on all the chromosomes in the genome and hence would probably give greater chance in yielding resistance mutants. There is no *a priori* bias against dominants.

Any mutant with selective disadvantage does have a finite chance of being lost, particularly if it is a dominant effect. The effects of this circumstance are minimized by testing stocks as rapidly as is compatible with generation of homozygotes. Perhaps, the main disadvantage of screen 3 is that it is not as genetically defined as screen 2. Such disadvantage might be quite serious, as has been learned recently from the genetic instability of almost all the mutants isolated in this study. Thus, to achieve full advantage of screen 3 in yielding stable resistance mutants, a provision should be made for the immediate location and stabilization of the newly isolated mutants by means of genetic crosses. Unfortunately such a provision was not made in the present study since the instability of the mutants was not recognized until late in the course of the investigation.

Table 20 summarizes the conditions and the results of the different selection screens applied in this study. As mentioned above, mutants were isolated only at low FUDR concentration ($1.5 \times 10^{-6} \text{M}$), thus it appears to be the main factor in the failure of screen 1 and early trials with screen 2.

The failure of the various screens to produce FUDR resistance mutants at higher doses of the analogue has precedent in the literature. Freed and Mezger-Freed (1973) reported that, in haploid frog cells, one-step resistance to the thymidine analogue, BUdR (which causes mis-coding by incorporation into DNA, after being phosphorylated initially by thymidine kinase) can only be obtained to $5 \times 10^{-5} \text{M}$ or less BUdR. Higher levels of resistance can be obtained by a subsequent increase in the concentration of the analogue (10^{-3}M). Thus it seems that the lower resistance is an obligate requirement to obtain higher resistance. It is interesting to note, however, that the resistance was found to be of different nature in the two cases. At low concentration the mutation was associated with impairment in the transport system while at higher concentration the mutation was due to an additional deficiency in thymidine kinase.

Deficiency in transport systems does not appear to be a mandatory intermediate stage in obtaining highly analogue resistant mutants in other cell types. Orkin and Littlefield (1971) have demonstrated the inability to produce mutants highly resistant to aminopterin by a single step in cultured Chinese hamster cells, but reported that neither the highly nor the slightly resistant mutants isolated were deficient with regard to their uptake of the analogue.

IV. NUTRITIONAL CHARACTERIZATION OF THE MUTANTS

To characterize the resistant mutants and to explore differences in the resistance phenotype, their response to increased FUDR concentration was tested in the presence or absence of either thymine or thymidine.

A. Dose response in the absence of thymine

Since all mutants were isolated on thymine containing medium, their response to the removal of this base was studied to test the possibility that they were defective in the enzyme thymidylate synthetase. In this case it is assumed that such a mutant would be unable to survive the absence of thymine.

Initially, newly-hatched larvae from five mutant strains from screen 2 were transferred to thymine-free defined medium containing different concentrations of FUDR. *Am Or*⁺ (strain 1, Table 3) was used as a control; however, because of the difference in the genetic background between this strain and the mutants selected in screen 2, another strain, *C92-2*, isolated by Naguib (1976), was also introduced as a control. This latter strain contains a recessive lethal like all the resistant mutants from screen 2 and, having been obtained without selection from the same screen, would also be expected to have a similar genetic background.

Table 21 shows the results of this larval transfer experiment. Removal of thymine has no clear effect on survival to eclosion of any strains tested. Thus it can be assumed that none of these strains

Table 21. Percent Survival to Ecdysis and to Pupariation* of Screen 2 Mutant Larvae Grown on Defined Medium Containing Different Concentration of Fluid

Strain	Fluid Concentrations (M)									
	2.3×10^{-7}	5×10^{-7}	6.06×10^{-7}	7.34×10^{-7}	8.89×10^{-7}	1.08×10^{-6}	1.31×10^{-6}	1.58×10^{-6}	2.3×10^{-6}	5×10^{-6}
<i>Am Oc⁺</i>	51 (54)	47 (53)	53 (66)	28 (63)	26 (51)	10 (48)	2 (35)	0 (10)	0 (4)	0 (0)
<i>C98-2</i>	47 (57)	58 (72)	44 (59)	24 (51)	13 (30)	1 (19)	0 (3)	0 (8)	0 (0)	0 (0)
<i>16</i>	51 (59)	58 (98)	28 (65)	35 (50)	27 (60)	25 (48)	12 (28)	0 (3)	0 (0)	0 (0)
<i>228</i>	36 (48)	49 (61)	42 (49)	44 (61)	44 (56)	37 (57)	26 (43)	2 (19)	7 (21)	0 (0)
<i>219</i>	45 (48)	37 (40)	30 (39)	23 (43)	28 (51)	17 (43)	13 (34)	1 (14)	3 (21)	0 (0)
<i>2223</i>	46 (49)	41 (52)	34 (55)	28 (37)	27 (38)	24 (30)	12 (31)	7 (22)	3 (17)	0 (0)
<i>2217</i>	49 (59)	64 (72)	36 (50)	52 (72)	36 (60)	17 (43)	13 (37)	2 (25)	1 (16)	0 (0)

* Each datum is based on a minimum of three replicates of 50 larvae.

* Pupariation is shown between parentheses.

is deficient in thymidylate synthetase.

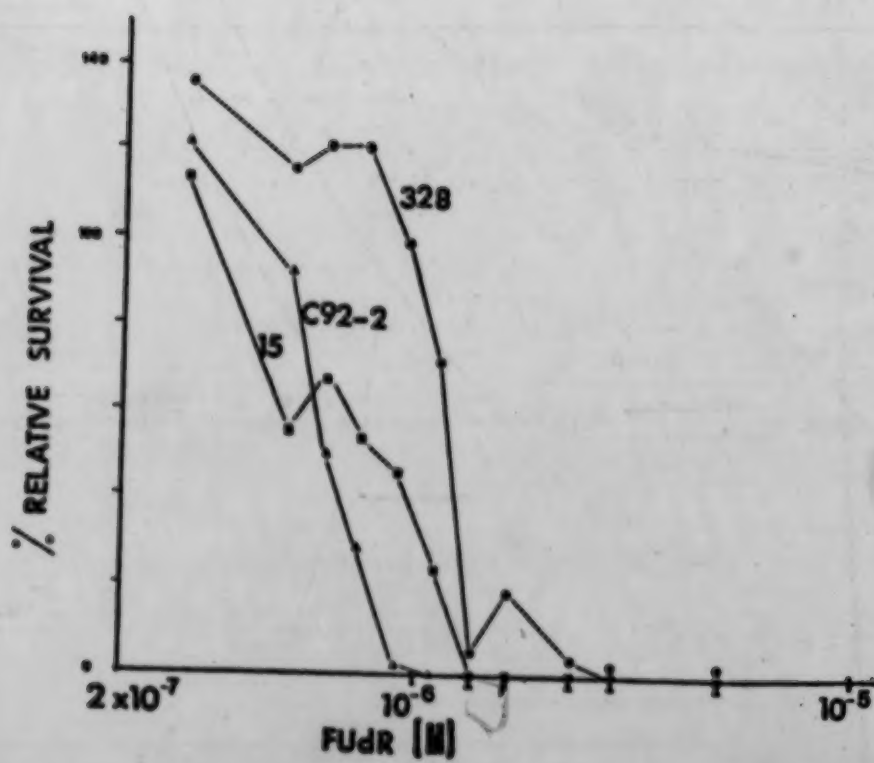
The five mutant strains tested are clearly more resistant to FUDR than either *Am Or*⁺ or *C92-2*. At 1.08×10^{-6} M FUDR, between 12% and 26% of the mutant larvae reached adulthood compared to 2% for *Am Or*⁺ and none for *C92-2*. Larvae of the four mutant strains; 328, 519, 2923 and 3317 eclosed at two FUDR concentrations (1.31×10^{-6} and 1.58×10^{-6} M) where no survival was observed for either *Am Or*⁺ or *C92-2*. Strain 328 showed the greatest resistance among the mutants. The overall resistance, nonetheless, is relatively low.

Strain *C92-2* showed similar sensitivity to FUDR as wild type *Am Or*⁺ if not more; hence it seemed adequate for use as a control for screen 2 mutants.

Some dose response curves (Fig. 8) for the mutants are sigmoidal (328), but others are less so (15). In strain 15 resistance appears to be a function of a sub-population consisting of about 50% of the larvae. The dose response curve of 328 also indicates the probable existence of highly resistant rare "segregants" in this strain. Such pattern of behaviour necessitates the use of response to several FUDR doses when estimating and comparing resistance of the different mutant strains.

In spite of the more accurate measurements obtained by larval transfer experiments in estimating the response to variety of dietary supplements, it is time-consuming. Thus the adult oviposition method (see *MATERIALS AND METHODS*) was adopted to speed up the characterization of the 14 mutants.

Figure 8. Relationship between the percentage relative survival of C92-2, 15 and 328 mutant larvae on defined medium and the concentration of FUDR. Data is normalised to survivorship of the individual strains in the absence of FUDR.



Tables 22 and 23 show the relative productivity of mutants isolated in screen 2 and 3, respectively, on defined medium containing different concentrations of FUDR in the absence of thymine. Productivity for all strains, both mutants and controls, is in the range of 5.8-11.0 progeny/female/day (p/f/d) (average = 8.4) for the stocks bearing the recessive lethals (screen 2, Table 22) and from 6.4-15.8 p/f/d (average = 11.1) for the ostensibly homozygous stocks (screen 3, Table 23). The fact that all produce substantial numbers of offspring indicates that none is of the conventional *thy*⁻ phenotype. All mutants except one tolerate 7.34×10^{-7} M FUDR slightly better than controls. The most striking mutant isolated, A37, produced 56 times as many offspring as the control (Table 23) while the rest of the mutants gave offspring 2 to 15 times greater than their controls (see Tables 22 and 23). The difference becomes more obvious at 1.08×10^{-6} M FUDR. A37 is almost 200 times as productive as the control (see Table 22). The rest of the mutants either exhibit similar or somewhat higher resistance than controls.

The comparatively minor differences observed between these results and those obtained by larval transfer experiments (c.f. Tables 21 and 22) indicates the adequacy of adult oviposition for quick characterization of the mutants.

Occasional adults from control strains surviving on high FUDR doses almost always prove sterile even when subsequently transferred to dead-yeast medium, whereas mutant flies are generally fertile. Although this has not been used extensively, it could be applied so as to clean up the "adult oviposition" assay further, since

Table 22. Relative Productivity of Screen 2 Mutant Females (1) Ovipositing Defined Medium Containing Different Concentrations of FdR

Strain	NONE	FdR Concentrations [M]				Absolute Average Productivity(2)
		7.54×10^{-7}	1.08×10^{-6}	1.31×10^{-6}	1.58×10^{-6}	
C92-2	100 (30)	5.4 (30)	1.1 (30)	0 (30)	0 (30)	9.2
16	100 (35)	10.3 (30)	0.7 (30)	0.7 (25)	0 (30)	10.3
17	100 (30)	18.0 (30)	10.6 (25)	0.3 (30)	1.8 (30)	6.5
63	100 (40)	17.0 (35)	4.3 (35)	1.8 (35)	0 (35)	7.9
68	100 (20)	11.0 (20)	0.6 (20)	0.4 (20)	0 (20)	6.4
68	100 (35)	11.7 (25)	1.9 (30)	0.6 (30)	1.1 (30)	7.8
818	100 (30)	14.2 (25)	1.7 (25)	1.7 (25)	0.4 (25)	5.8
819	100 (45)	12.1 (35)	5.0 (40)	0 (40)	0.7 (40)	10.2
8223	100 (45)	17.2 (40)	3.2 (40)	0.7 (40)	0 (40)	9.4
8217	100 (35)	13.0 (30)	0.3 (30)	0.5 (30)	0 (30)	11.0

¹Number of female parents is shown in parentheses.

²Estimated as number of progeny/female/day in the cultures without FdR.

Table 23. Relative Productivity of Screen 3 Mutants Females.⁽¹⁾ Ovipositing on Defined Medium Containing Different Concentrations of FdR

Strain	FDR Concentrations [M]				Absolute Average Productivity(2)
	NONE	7.34×10^{-7}	1.08×10^{-6}	1.31×10^{-6}	
Am Cr ⁺	100 (45)	1.0 (45)	0.1 (45)	0 (45)	10.9
427	100 (40)	56.0 (35)	19.3 (35)	4.0 (35)	6.4
208	100 (40)	8.9 (35)	0.1 (35)	0 (30)	11.7
148	100 (50)	15.0 (45)	0.6 (50)	0 (45)	10.9
273	100 (30)	12.3 (30)	0.4 (25)	0 (25)	15.8
290	100 (45)	5.0 (40)	0.1 (40)	0 (40)	11.5

¹Number of female parents is shown in parentheses.

²Estimated as number of progeny/female/day in the cultures without FdR.

the "escaper" flies are rather more common using this method than in larval transfer experiments.

B. Dose response in the presence of thymine

Since all mutants were selected in the presence of thymine, comparison of their response to increased doses of FUdR would probably be expected to give maximal discrimination between the mutants and the controls. The relative productivity of mutant females on defined medium containing thymine ($5 \times 10^{-3}M$) and different concentrations of FUdR is shown in Tables 24 and 25.

Under these conditions A37 was, once again, distinguished among the mutants by its high level of resistance. At $1.08 \times 10^{-6}M$ and $1.58 \times 10^{-6}M$ FUdR it showed 30 and 20 fold, respectively, increased resistance over the control (see Table 25). The rest of the mutants exhibited different ranges of resistance, according to their genetic origin. Mutants from screen 3, with the exception of A37, showed resistance ranging almost 3 to 6 times greater than that observed for controls at $1.08 \times 10^{-6}M$ FUdR, 290 being the most resistant among this group.

Mutants isolated in screen 2 (Table 24) were less resistant than those of screen 3 (Table 25) when compared to their control, C92-2, at $1.08 \times 10^{-6}M$ FUdR or higher. C92-2 is, it seems, resistant to FUdR in the presence of thymine but not in its absence. It should be remembered that C92-2 stock was not the standard by which the mutants were selected originally and it must be assumed that its resistance is an inconvenient chance happening. Certainly, several thousands of less

Table 24. Relative Productivity of Screen 2 Mutant Females (1) Ovipositing on Defined Medium Containing Different Concentrations of FUDR and Thymine ($5 \times 10^{-5}M$)

Strain	NONE	FUDR Concentration [M]				Absolute Average Productivity(2)
		7.34×10^{-7}	1.08×10^{-6}	1.31×10^{-6}	1.58×10^{-6}	
C92-2	100 (45)	37.2 (35)	11.1 (40)	3.4 (35)	2.5 (40)	6.0
16	100 (35)	41.3 (35)	10.9 (35)	7.9 (35)	1.2 (20)	10.2
17	100 (35)	52.9 (35)	14.7 (35)	1.5 (35)	0.4 (25)	6.4
63	100 (40)	88.3 (40)	39.4 (40)	5.7 (40)	0.9 (40)	4.5
66	100 (20)	68.2 (20)	6.7 (20)	8.2 (20)	2.2 (20)	3.4
68	100 (35)	27.8 (35)	8.8 (35)	7.4 (35)	0.4 (35)	7.9
338	100 (30)	60.8 (30)	11.6 (30)	4.2 (30)	1.5 (30)	5.2
519	100 (45)	60.4 (45)	26.3 (35)	7.5 (45)	3.7 (45)	9.1
8023	100 (40)	69.1 (45)	16.3 (45)	8.0 (45)	2.2 (45)	9.3
3317	100 (35)	39.9 (30)	14.1 (35)	10.2 (35)	1.0 (25)	9.9

¹Number of female parents is shown in parentheses.

²Estimated as number of progeny/female/day in the cultures without FUDR.

Table 25. Relative Productivity of Screen 3 Mutant Females⁽¹⁾ Ovipositing on Defined Medium Containing Different Concentrations of FUDR and Thymine ($5 \times 10^{-3}M$)

Strains	NONE	FUDR Concentrations [M]			Absolute Average Productivity ⁽²⁾
		7.34×10^{-7}	1.08×10^{-6}	1.31×10^{-6}	
<i>Am Or</i> ⁺	100 (45)	52.8 (45)	2.5 (45)	1.4 (45)	1.1 (45)
A37	100 (40)	101.4 (30)	76.3 (40)	38.6 (35)	22.6 (40)
109	100 (35)	46.0 (40)	13.3 (40)	1.0 (40)	1.0 (35)
146	100 (45)	43.9 (50)	6.7 (50)	2.7 (60)	0.7 (50)
173	100 (30)	22.9 (25)	13.8 (30)	4.3 (30)	1.2 (30)
280	100 (45)	38.6 (45)	15.8 (45)	4.6 (45)	3.1 (40)
					10.3 ⁻

¹Number of female parents is shown in parentheses.

²Estimated as number of progeny/female/day in the culture without FUDR.

resistant strains were discarded in the course of screen 2; *C92-2* was not among them. Differences between the resistance of *C92-2* and the mutant strains except *58*, can be better demonstrated at 7.34×10^{-7} M FUDR. The resistance factor ranges from approximately 1.1 for *3317* to 2.4 for *53*. All strains, *58* and *C92-2* included, are resistant by comparison with *Am Or⁺*, which was the original standard.

C. Dose response in the presence of thymidine

Resistance which is generated by limitation of access of FUDR to the cell, rather than by specific means which circumvent its effect on thymidylate biosynthesis, should exhibit itself in the presence of thymidine. Resistance generated by rapid conversion of FUDR to FU, in contrast, should lower the effectiveness of thymidine as antidote. Hence the effects of FUDR in the presence of thymidine were tested. Tables 26 and 27 show the relative productivity of mutant females on defined medium containing different concentrations of FUDR in the presence of thymidine (5×10^{-3} M).

Thymidine seems to accentuate the difference in resistance between the mutants isolated in screens 2 and 3. With the exception of strains *17* and *53*, only marginal differences are observed between the control (*C92-2*) and the mutants isolated in screen 2 at 1.77×10^{-5} M FUDR (see Table 26). In this case, the same is true if comparison is made with *Am Or⁺*. On the other hand, at the same concentration, mutants isolated in screen 3 were 3.2 to 5.7 fold more resistant, than the control (see Table 27). These differences were still evident at 3.16×10^{-5} M FUDR. In fact, at 1.77×10^{-5} M FUDR mutants of screen 3 along with *17* from

Table 26. Relative Productivity of Screen 2 Mutant Females⁽¹⁾ Ovipositing on Defined Medium Containing Different Concentrations of FldR and Thymidine (5×10^{-5} M)

Strain	NONE	FldR Concentration [M]						Absolute Average Productivity ⁽²⁾
		10^{-5}	1.77×10^{-5}	2.37×10^{-5}	3.16×10^{-5}	5.6×10^{-5}	10^{-4}	
C92-2	100 (40)	18.3 (15)	15.7 (40)	3.5 (40)	0	0	0	5.6
15	100 (50)	33.1 (15)	5.3 (25)	3.0 (25)	0.5 (30)	0	0	10.2
27	100 (30)	36.0 (20)	87.4 (30)	30.4 (30)	13.4 (35)	0.5 (30)	0	6.7
63	100 (35)	25.8 (20)	42.5 (30)	19.5 (30)	2.7 (35)	0	0	4.3
66	100 (20)	37.0 (10)	27.1 (20)	6.9 (20)	2.0 (20)	0	0	5.1
58	100 (25)	14.5 (15)	18.4 (25)	7.7 (25)	2.0 (30)	0.3 (30)	0	6.0
228	100 (25)	27.4 (15)	17.4 (20)	9.0 (20)	7.2 (25)	0	0	4.7
519	100 (25)	61.5 (25)	14.2 (20)	2.8 (20)	3.8 (35)	0	0	5.3
2923	100 (20)	57.9 (20)	17.0 (15)	4.5 (15)	1.8 (25)	0	0	6.7
3317	100 (25)	90.2 (25)	25.0 (20)	8.3 (20)	2.1 (30)	0	0	5.7

¹Number of female parents is shown in parentheses.

²Estimated as number of progeny/female/day in the culture without FldR.

Table 27. Relative Productivity of Screen 3 Mutant Females (1) Ovipositing on Defined Medium Containing Different Concentrations of FldR and Thymidine ($5 \times 10^{-5}M$)

Strain	NONE	FldR Concentrations (M)					Absolute Average Productivity (2)
		10^{-5}	1.77×10^{-5}	2.37×10^{-5}	3.16×10^{-5}	5.6×10^{-5}	
<i>Am Or⁺</i>	100 (20)	68.8 (25)	16.0 (20)	12.5 (20)	6.7 (30)	0 (30)	12.2
<i>A37</i>	100 (25)	73.1 (30)	73.0 (25)	48.5 (25)	33.9 (35)	1.1 (35)	3.9
<i>100</i>	100 (40)	73.6 (25)	65.7 (35)	41.6 (35)	21.0 (35)	0.7 (35)	12.0
<i>160</i>	100 (30)	86.4 (25)	71.3 (25)	31.1 (30)	20.0 (40)	0.5 (40)	9.0
<i>173</i>	100 (25)	61.5 (20)	51.5 (25)	37.3 (30)	25.5 (30)	0 (25)	13.8
<i>200</i>	100 (30)	91.2 (25)	89.8 (25)	41.1 (25)	16.8 (35)	0 (30)	6.8

¹Number of female parents is shown in parentheses.

²Estimated as number of progeny/female/day in the cultures without FldR.

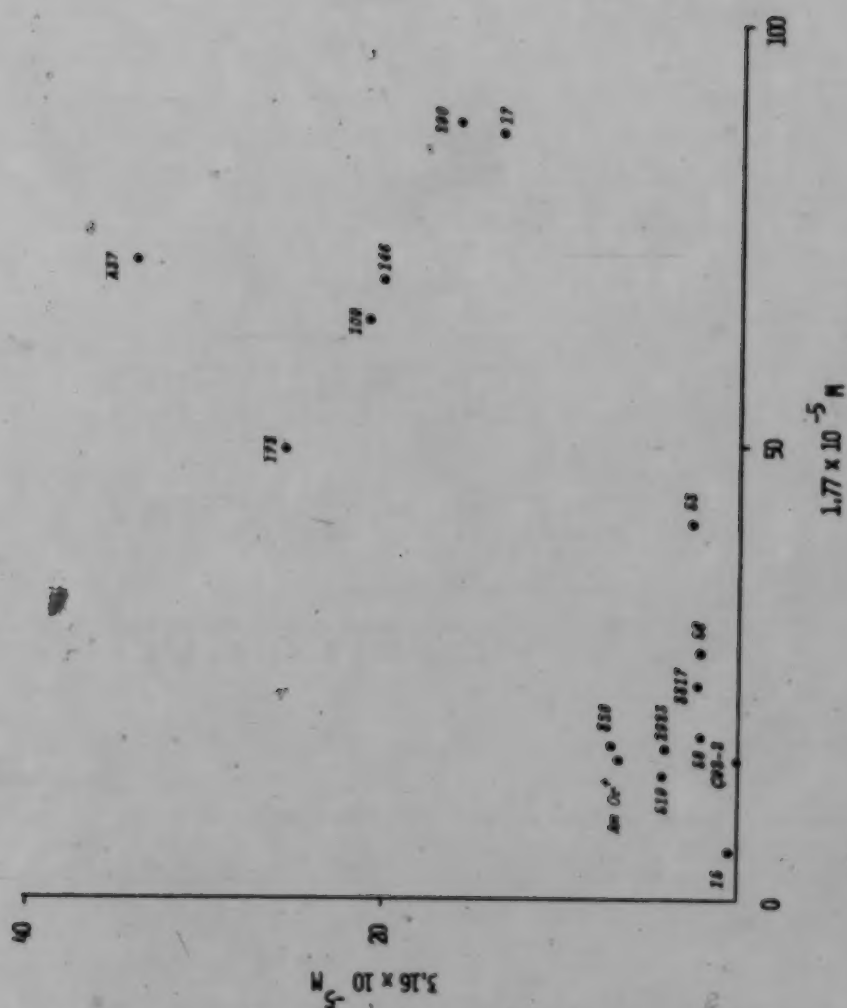
screen 2 were comparable in resistance to the highly resistant strain, A37. However, the difference between these strains and A37 was evident at the higher concentrations of 3.16×10^{-5} M FUDR.

Thus, in the presence of thymidine, the mutants can be grouped in three major classes by comparing their relative productivity at two concentrations of FUDR. The first group would include the highly resistant A37. A moderately resistant class would include strain 17 from screen 2 mutants and the rest of the mutants from screen 3. The rest of the mutants would fall into a class of marginal resistance, with strain 53 perhaps falling between the last two classes of resistance. Fig. 9 illustrates such classification.

Despite the fact that A37 is still the most clearly resistant mutant, it is relatively less striking on thymidine than in the other tests. This could be due to its reduced average productivity (3.9 pfd) on thymidine (without FUDR) as compared with the control (*Am Or⁺*, 12.2 pfd) or its own behavior under different nutritional conditions, 6.4 pfd on defined medium alone and 7.7 pfd on thymine, (see Tables 23 and 25). The suspicion that this strain is sensitive to thymidine has been partly confirmed by preliminary experiments at higher thymidine concentrations.

A37 differs from the rest of the mutants in so far as it is apparently a more stable mutant strain, having consistently retained its resistance, in the absence of the analogue. The rest of the mutants are less stable and require periodical reselection to retain their resistance. Similar instabilities have been reported for mutants

Figure 9. The relationship between relative productivity for the various strains (mutant and control) at two different FUDR concentrations in the presence of $5 \times 10^{-3} \text{M}$ thymidine.



resistant to juvenile hormone analogues in *Drosophila* (Arking and Vlach, 1976), FUdR resistant mutants in neuroblastoma (Baskin *et al.*, 1975) and several drug resistances in bacteria (Rosset, personal communication). The phenomenon is also widely observed among temperature sensitive mutants in *Drosophila*. Instability in the FUdR resistant stocks could arise from natural selection of modifiers in the strains or because the mutant genotypes have not become fixed, as well as from the more conventional explanations, such as back-mutation or unequal crossing over between duplications.

The overall low relative resistance of the mutants is compatible with the selection screen applied. However, more highly resistant mutants could have been isolated by the screen, but were not. Nor did more stringent screens produce positive results, so that it seems likely that highly resistant mutants are rare. Two-step high-level resistance remains a possibility (see Orkin and Littlefield, 1971; Freed and Mezger-Freed, 1973).

Low level resistance of *Drosophila* mutants to analogues was also reported by Duke and Glassman (1968) for FU, by Sherald and Wright (1974) for methyl dopa and Arking and Vlach (1976) for juvenile hormone analogues. No instances of very high levels of analogue resistance have yet been reported from *Drosophila*.

V. GENETIC CHARACTERIZATION OF THE MUTANTS

None of the mutant strains isolated demonstrated startlingly high levels of FUdR resistance. The resolution and ease of genetic analysis

of resistance is clearly dependent upon the level of resistance. In consequence, only preliminary genetic characterization of some of the mutants isolated in this investigation has been carried out; some of the results are reported below.

A. Complementation Studies

Strains isolated in screen 2 carry recessive lethals with regard to the second chromosome, so that all flies have *Curly* (*Cy*) wings. Crosses between the different strains indicate that in every case recessive lethal genes are non-allelic, since all possible crosses produced offspring with normal (non-*Curly*) wings when grown on yeast-sucrose medium.

An association between recessive lethality and resistance would be quite puzzling if significant. However, taking into consideration that the recessive lethals accounted for 60% of the total strains tested, it is possible that the association arose by chance. Nevertheless, it seemed possible that recessive lethals do themselves confer dominant resistance as heterozygotes. If such were the case there must be several genes on the second chromosome capable of generating resistance mutations of this kind. However, growing the same crosses on defined medium with FUDR (see below) indicates that this is not a correct interpretation.

Reciprocal crosses between mutant strains (taken pairwise) grown on defined medium containing FUDR ($1.5 \times 10^{-6}M$) and thymine ($5 \times 10^{-3}M$), yielded no viable offspring. This result suggests three things. The mutants are not dominant; they are not sex-linked; and

finally, they are not allelic.

First generation crosses of screen 3 mutants in the mapping experiments, when grown on FUDR-containing medium, also fail to produce offspring, further reinforcing the conclusion that the mutants are neither dominant nor sex-linked.

B. Mapping studies

Attempts to map resistance were focused primarily on mutants from screen 3, because of their stronger expression of resistance and their apparent genetic simplicity. The initial attempts were made with A37 and 290 as representatives of the group. Virgin females from a mutant strain were mated to multiply heterozygous $Cy/2^4$; $Ly\ Sb/3^4$ males (2^4 and 3^4 identify autosomes from the same mutant strains). Similar crosses were later repeated with heterozygous males carrying Pm instead of Cy since Cy ; $Ly\ Sb$ has low viability and it was also hoped to map mutants from screen 2, where Cy was already present. Strain 146, 173 and 290 from screen 3, as well as strain 519 as a representative of mutants from screen 2, were used in this mapping scheme. The results are shown in Table 28.

A37 data are not shown in Table 28 because fertility problems arose during the crossing procedure.

The results from the F_2 suggest that 290 as well as 173 are probably third chromosome mutations while 146 is probably located on the second chromosome.

Table 28. Genotype Distribution of Offspring from Crosses Between Heterozygous Male Mutants and Homozygous Female Mutants Grown on Defined Medium Containing FUDR ($1.3 \times 10^{-6}M$) and Thymine ($5 \times 10^{-3}M$) and on Yeast-Sucrose Medium⁽¹⁾

Female Genotype	Male Genotype	Offspring Genotype ⁽²⁾			
		M/2 ; M/3	M/2 ; 3/3	2/2 ; M/3	2/2 ; 3/3
146	Pm/2 ; Ly, Sb/3	3 (-)	2 (-)	18 (-)	28 (-)
173	Pm/2 ; Ly, Sb/3	3 (22)	13 (39)	8 (44)	29 (48)
290	Pm/2 ; Ly, Sb/3	1 (-)	11 (-)	1 (-)	13 (-)
	Cy/2 ; Ly, Sb/3	4 (227)	81 (241)	4 (251)	18 (330)
510	Pm/2 ; Ly, Sb/3	52 Cy ; Ly, Sb (367)	74 Cy (583)	0 (0)	0 (0)
		4 Pm ; Ly, Sb (594)	21 Pm (289)		

¹Shown in parentheses. The sign (-) indicates data not available.

²2 represent second chromosome, 3 third chromosome and M markers on either chromosome.

Since mutants from screen 2 all carry recessive lethals on the second chromosome, no homozygous second chromosome could be obtained in this mapping protocol. Also, because of the lethality of the *Pm/Cy*, only four progeny classes are expected in this cross, $Cy/2^A; 3^A/3^A; Cy/2^A; Ly\ Sb/3^A; Pm/2^A; 3^A/3^A$ and $Pm/2^A; Ly\ Sb/3^A$. Equal distribution of offspring between the four classes would suggest that the resistance mutation is dominant and carried on either the second or the third chromosome. Appearance of *Cy* or *Pm* offspring only would indicate the recessiveness of the mutation and that it is on the third chromosome. The results in Table 28 suggest that 519 is recessive and is located on the third chromosome; however, it seems that the SMS chromosome *per se* or gene(s) on it enhance the expression of resistance. This contention could also be supported by crosses between 290 and *Cy*, *Ly Sb*. In both cases homozygous third chromosome flies are in excess in the presence of SMS.

Thus it seems probable that chromosomal locations can be assigned to the factors generating resistance; by extension it is not unreasonable to suggest that precise genetic localizations will eventually be possible. Nonetheless, Glassman and Duke (1968) encountered difficulties in extending mapping of FU-resistance factors below the level of chromosomal assignment and their problems must be borne in mind. It is also premature to suggest that the above data necessarily provides a definitive genetic description of the mutant strains, because careful analysis of the dose responses of the strains might well demonstrate them not to be monophasic, as was shown to be the case for 519 (see Fig. 8). Were this the case the method would identify the genetic

factors responsible for the more resistant members of the population, but would not describe the factors responsible for the inherent population variability. Clearly, a proper description of the genetic composition of a strain of that kind will require considerable refinement of genetic techniques.

GENERAL CONCLUSIONS

The present investigation is the first systematic attempt to utilize nutritional manipulation together with genetic analysis to study the effect of a nucleoside analogue, in this case 5-fluoro-2'-deoxyuridine, upon development of a higher eukaryote. The ultimate aim was to explore the possibility of screening for resistant mutants for use in studying gene function and regulation in the fruit fly.

This study establishes beyond doubt the potential sensitivity of nutritional manipulation as a tool in analyzing the effects of compounds upon whole multicellular organisms. The responses observed are exceedingly close to those found using cell cultures and even correspond reasonably well with results obtained from studies on cell extracts. The possibility that compounds are changed by incorporation into a complex nutrient medium does not, in this study, appear to be of great significance; nor does it appear that the whole organism itself modifies the essential action of the analogue.

Not only does the effect of FUDR itself correspond with that reported in bacteria and vertebrate cells in culture, but so do its interactions with several pyrimidine-containing compounds. This detailed correspondence suggests that the somewhat unexpected effects of deoxycytidine can be interpreted as properties of nucleotide metabolism in the fly and not as an outcome of the unusual technique by which they were discovered. Similarly, effects of the natural pyrimidine or purine bases or nucleosides, which have not been observed in

other systems, can be interpreted as aspects of the fly's metabolism.

In contrast to the studies of nutritional manipulation, the genetic analysis of FUdR resistance was much less successful. No resistant mutants were isolated at the higher FUdR concentrations used and none of those isolated at low dose ($1.5 \times 10^{-6} \text{M}$) exhibited a high level of resistance. It has been argued that such highly resistant mutations could not arise in their animal cells without a "prior modification" in cellular metabolism (Freed and Mezger-Freed, 1973). These authors suggest the modification is probably a low level hereditary resistance, but do not rule out the possibility that it is a transmissible change of metabolic state unaccompanied by gene mutation, since they use a cell culture system.

It is entirely possible that two-step selection is a requirement to obtain mutations resistant to high doses of the analogue, as is the case in vertebrate cells in culture. It has to be mentioned, however, that F_2 crosses between some strains obtained in this study (A37, 146, 290, 2923 and 3317) did not segregate individuals with strikingly higher resistance than their parents. These crosses were not carried out with extreme rigour (for example, only one FUdR dose was used), so that it is entirely possible that double mutants which are highly resistant could be produced even from already available materials. However, if the "metabolic state" hypothesis were proven correct, then two step selection would not work unless the shift in "state" were transmissible through germ lines, which seems unlikely.



Three main categories of mechanisms have been reported for resistance to FUdR. The first category is related to deficiencies or alterations in the enzymes involved in the activation of FUdR or its derivatives, the most frequent enzyme being thymidine kinase. The second category is related directly to *de novo* dTMP biosynthesis and mutants of this kind are shown to result from either elevation in the synthesis of the enzyme thymidylate synthetase or alterations in its substrate specificity in such a manner that the enzyme has preference for the natural substrate (dUMP) over its fluorinated analogue (FdUMP). In addition, it is postulated that thymidylate synthetase negative mutants might also be FUdR resistant in the presence of thymine, by analogy with the demonstrated anti-folate drug resistance of such mutants in bacteria. The third category, which is quite common, involves modifications in the transport system so that FUdR cannot gain entry to the cell.

None of the mutant strains isolated in this study seems to lack thymidine kinase, since all responded to the presence of thymidine to overcome FUdR toxicity. These results are rather strange in view of the high frequency of occurrence of such mutations in cell culture and bacteria. In addition, *Drosophila* is capable of pyrimidine *de novo* biosynthesis and, hence, it might be expected that the absence of a salvage enzyme would not affect its viability. However, it could be argued that the *de novo* biosynthetic capability of *Drosophila* is limited or that it occurs in excess in specific tissues in the body of the fly and is transported to other tissues which synthesize less than their full requirements. In these cases, the salvage enzymes

might be of vital importance, either in recycling or in cell-cell transfer of pyrimidines. The transportation model is of some interest. It is generally assumed that pyrimidines are transported among the different tissues of the fly in the nucleoside form. If thymidine, rather than uridine, is the obligate precursor for dTMP in recipient tissues, then thymidine kinase would be essential.

Support for the proposition that some tissues are specialized for pyrimidine biosynthesis is found in mosaic studies of Falk (personal communication) which suggest that pyrimidine biosynthesis in *Drosophila* larvae is localized primarily in the fat body.

On the other hand, FUDR itself could impose critical limitations on *de novo* pyrimidine biosynthesis in the presence of a thymidine kinase mutant, even if all tissues have sufficient capacity for *de novo* biosynthesis. FUDR is converted to FdUMP by thymidine kinase, but a proportion of it is cleaved to FU by thymidine phosphorylase. In the absence of thymidine kinase, extra FU should be produced. FU in turn can be converted to FUTP, a known feedback inhibitor of *de novo* pyrimidine biosynthesis (see, for example, Lacroute, 1968). If *Drosophila* is sensitive to such feedback inhibition by FUTP, the loss of thymidine kinase by mutation would accentuate the effects of FUDR, *via* FUTP, on *de novo* pyrimidine biosynthesis and possibly cause death rather than resistance.

It has been suggested that imaginal discs of *Drosophila* are relatively isolated from exogenous pyrimidine sources and depend upon endogenous biosynthesis for normal development during the 18 hrs. following

pupariation (Falk, submitted for publication; see also, Falk and Nash, 1974b). The period might be a critical target time for the action of FUdR in a thymidine kinase mutant, even if the larva could survive using maternal pyrimidines for some time, which is at least a possibility. Thus kinase mutants would die as puparia. There is a certain amount of evidence supporting this possibility; uridine does improve survival of larvae grown in the presence of FUdR and thymidine (see Table 7) until pupariation, but not to the imaginal stage.

When both hypotheses are considered, the inhibition of *de novo* biosynthesis seems to be more likely the case. The synthesis of dTMP generally proceeds *via* two pathways: it can either be synthesized from dUMP by thymidylate synthetase or from thymidine by thymidine kinase. The suggested model explaining the absence of thymidine kinase negative mutants because the enzyme *per se* is required even in the absence of FUdR, implies that only one route, *via* thymidine kinase, is available to the fly. The models in which endogenous *de novo* biosynthesis is affected in the presence of FUdR applies even when both pathways are functional.

The detailed version of the second model, in which thymidine kinase negative mutants become FUdR sensitive as pupae, suggests that selection for mutants deficient in thymidine kinase might be performed by the use of medium shift experiments. Potential mutants having prolonged survival on FUdR could be transferred to rich nutrient medium to maximize nutrition and dilute FUdR. This procedure might overcome the suggested inhibition of the *de novo* pyrimidine biosynthesis during the critical period after pupariation. This screen would, of course,

also identify other resistant mutants. It apparently will not pick up auxotrophic mutants (Vyse, personal communication), although it might be expected to do so.

The present results also eliminate the possibility that any of the mutants exhibits total absence of thymidylate synthetase, since all strains grow in the absence of a dTMP source. Likewise, a total loss of enzyme sensitivity to FdUMP is ruled out, because all mutant strains responded positively to thymidine in the presence of FUDR.

These results could be due to the rarity of such mutant types, the number of strains tested having been fairly low. Thymidylate synthetase mutants might also have been selected as auxotrophs, but none has been found, perhaps for the same reason.

It could also be argued that the precise physiological requirements for the selection of this type of mutant are not met in the present study. The simplest approach to the production and assessment of synthetase negative mutants would probably be to locate the gene for thymidylate synthetase genetically, before searching for the mutants, since the synthetase assay is fairly well established, as is the aneuploid method for gene localization. The same methodology could be employed for thymidine kinase; however the enzymological methods for thymidine kinase assay in *Drosophila* are much less developed than those for thymidylate synthetase.

Elevation in activity of thymidylate synthetase is another possibility for explaining FUDR resistance. In such mutants the intracellular concentration of dTMP could be elevated and this might

render them hypersensitive to exogenous sources of dTMP.

Strain A37 showed a relatively higher level of resistance when compared with the other strains. Its productivity on thymidine- or thymine-containing media is less than that observed on defined medium alone, even in the absence of FUDR. Preliminary results using higher doses of thymidine seem to confirm this observation. Thus a plausible explanation for resistance in this mutant is that it has elevated activity for thymidylate synthetase.

Another possible mechanism for resistance to FUDR revolves around the uptake of the analogue. This mechanism of resistance could account for all or any of the mutants isolated in this study. However, in contrast to mutants which specifically overcome dTMP deficiencies, transport mutants might be expected to show resistance even in the presence of thymidine. On this basis, the mutants isolated can be divided into two groups: those which exhibit resistance (compared with wild-type) on thymidine, which are more likely to be transport mutants (all mutants from screen 3 and 17 and 53 from screen 2); and the rest, which lose their resistance on thymidine. This latter group (and possibly some or all from the first group) could be explained as leaky mutants in the synthetase or kinase genes, or as a slight elevation of synthetase activity, although perhaps the most satisfactory conclusion about the mutants is that their phenotypes are insufficiently striking to warrant definitive hypotheses.

The various suggested mechanisms could be tested further nutritionally. For example, resistance and sensitivity to high doses of

dietary thymidine might correlate with transport defects and with elevated synthetase activity, respectively. Nonetheless, *in vivo* tracer studies or *in vitro* enzyme assays would probably be the best approach to characterization of the mutants. However, the disappointingly low levels of resistance found in the mutants caution against undue optimism in their biochemical analysis.

In summary, although numerous difficulties are encountered in selection of FUDR-resistant mutants in *Drosophila*, the system might be improved by the introduction of some modifications, among them medium shift experiments, two-step selection, and more refined genetic manipulation of the resistant strains (as suggested in *RESULTS AND DISCUSSION* part III). Alternatively, the whole problem could be inverted, mutants affecting particular enzymes being sought first and then tested for resistance.

Clearly tests of these modifications have to be performed before a final judgement on the adequacy of analogue resistance, as a genetic and biochemical tool for the study of *Drosophila* nucleotide metabolism, can be made.

From a practical point of view, the system is an analogue of a "whole" human being and therefore probably involves factors pertinent to actual drug treatments, which are not simulated directly in tissue culture. It is reassuring to know that many of the responses of whole fruit-flies imitate the responses of tissue culture cells. However, the difficulty in generating resistant mutants probably illustrates the degree to which a whole organism must be conceived of as more than the

sum of its component parts and this proposition in itself is worth further study.

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APPENDIX

Theoretical Standard Deviations⁽¹⁾ Associated with
Selected Data Values at Minimum Sample Sizes
Shown in the Text

No. of Replicas	Corresponding Sample Size	Data Value (%) ⁽²⁾				
		1	5	10	25	50
2	60	1.2	2.8	3.9	5.6	6.4
3	90	1.0	2.3	3.2	4.6	5.3
6	180	0.7	1.6	2.2	3.2	3.7
7	210	0.7	1.5	2.1	3.0	3.4
8	240	0.6	1.4	1.9	2.8	3.2
9	270	0.6	1.3	1.8	2.6	3.0
10	300	0.6	1.3	1.7	2.5	2.9
13	390	0.5	1.1	1.5	2.2	2.5
17	510	0.4	0.9	1.3	1.9	2.2

¹Estimated as $\sqrt{\frac{pq}{n}} \times 100$, where p is frequency derived from data value, q = 1-p and n is the sample size.

²Standard deviations for data values above 50% are symmetrical with those below 50%.